University of Alberta

Hch1p Acts Differently From Its Homologue, Aha1p, in Regulating Sensitivity of Hsp90 to Hsp90 Inhibitors in Yeast

by

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ABSTRACT

Hsp90 is a molecular chaperone that plays a vital role in the regulation of oncoproteins, is targeted by small molecule Hsp90-inhibitors in cancer studies, and is functionally regulated by a cohort of proteins called co-chaperones.

We determined that deletion of the co-chaperone Hch1p, but not its homologue Aha1p, restores growth in yeast expressing the temperature-sensitive Hsp90 mutants, Hsp82p^{A587T} or Hsp82p^{G313S}. Strains expressing either of these Hsp90 mutants are hypersensitive to Hsp90 inhibitors. Sensitivity is reversed when *HCH1* is deleted. Over-expression of *HCH1* confers hypersensitivity to Hsp90 inhibitors in wild-type yeast, and *HCH1* deletion confers high resistance to these drugs. We also determined that Hch1p, but not Aha1p, is essential for growth of yeast expressing the temperature-sensitive mutant, Hsp82p^{E381K}. We conclude that the co-chaperone Hch1p plays an important role in regulating sensitivity to drugs that inhibit Hsp90 and regulates Hsp90 in a manner distinct from its homologue, Aha1p.

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in everything I do means to me, no matter if you are here with me or far away, I love you all very much. And to Kenton Nelson, you are my best friend and I could not imagine sharing my life with anyone else. You have made every step of this journey more enjoyable, and I will never be able to thank you enough for your continued support.

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LIST OF NOMENCLATURE

Abbreviation	Full Nomenclature
Δhch1	Knock out of a gene in yeast (HCH1 for example)
17AAG	17-*N*-Allylamino-17-demethoxygeldanamycin
17DMAG	17-Dimethylaminoethylamino-17-demethoxygeldanamycin
5FOA	5-Fluoroorotic acid
AMP	Ampicillin
APS	Ammonium persulfate
BSA	Bovine serum albumin
CYC	CYC terminator
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleotide triphosphate
DPBS	Dulbecco's phosphate buffered saline
DTT	Dithiothreitol
ECL	Enhanced chemiluminescence
EDTA	Ethylenediamine-tetraacetic acid
GA	Geldanamycin
GAL	Galactose
GEN	Geneticin
GLU	Glucose
GPD	GPD promoter

HCl	Hydrochloric acid
HIS3	Histidine biosynthesis 3 (Imidazoleglycerol-phosphate dehydratase)
His	Histidine tag
HRP	Horseradish peroxidise
Hsp100	Heat shock protein 100
Hsp70	Heat shock protein 70
Hsp82	Heat shock protein 82
Hsp90	Heat shock protein 90
Hsp90C	Heat shock protein 90 C-terminus
Hsp90M	Heat shock protein 90 M-terminus
Hsp90N	Heat shock protein 90 N-terminus
IgG	Immunoglobulin G
iH	Integrated at HIS3
iT	Integrated at TRP1
L-Arg	Arginine
L-Asp	Aspartic acid
L-Gly	Glycine
L-His	Histidine
L-Iso	Isoleucine
L-Leu	Leucine
L-Lys	Lysine
L-Met	Methionine
L-Phe	Phenylalanine

L-Ser	Serine
L-Thr	Threonine
L-Trp	Tryptophan
L-Ura	Uracil
L-Val	Valine
LB	Lysogeny broth
LiAc	Lithium acetate
LiAcTE	Lithium acetate, Tris base, EDTA solution
MIT	Massachusetts Institute of Technology
NaOH	Sodium hydroxide
NEB	New England BioLabs
NT	No tag
NVP	NVP-AUY922
O.D.	Optical density
PCR	Polymerase chain reaction
PEG	Polyethylene glycol
PMSF	Phenylmethanesulfonylfluoride
RAF	Raffinose
SDS	Sodium dodecyl sulphate
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
TAE	Tris base, Acetic acid, EDTA solution
TBS	Tris-buffered saline
TCA	Trichloroacetic Acid

TEF	TEF promoter
TEMED	Tetramethylethylenediamine
TPR	Tetratricopeptide repeat
Tris	Tris-(hydrocymethyl)aminomethane
TRP1	Tryptophan biosynthesis 1 (Phosphoribosylanthranilate isomerise)
URA3	Pyrimidine biosynthesis 3 (Orotidine-5'-phosphate (OMP) decarboxylase)
WT	Wild-type
YPD	Yeast peptone dextrose

CHAPTER ONE

Introduction

1.1 Protein Folding

One of the essential first steps towards understanding how cells work, from the most basic single cells to those that make up a human being, is to understand the activities and structures of the proteins within these cells. Within the cell and outside of it, proteins perform a vast range of functions that can give cells a selective advantage or can even be essential for cell survival. In order for most proteins to become functional they must fold to achieve their specific three-dimensional structure (Dobson et al. 1998; Hartl et al. 2011). In 1973, Christian Anfinsen proposed the 'thermodynamic hypothesis' which states that the three-dimensional structure of a native protein is determined by its linear amino acid sequence (Anfinsen 1973). Specifically, intramolecular interactions occurring between amino acids of a polypeptide chain drive protein folding (Anfinsen 1973). The hydrophobic intramolecular interactions are the major stabilizing forces that lead to the acquisition of a fully folded three-dimensional structure (Anfinsen 1973; Pace et al. 1996). In the setting of a cell, following translation, where protein is estimated to be at a concentration of 300 mg/mL, unfolded proteins are more likely to interact with other unfolded proteins due to protein crowding within the cell (Ellis 2001; Hartl et al. 2011). These improper hydrophobic protein-protein interactions lead to protein aggregation and abolish protein function (Ellis et al. 1991; Ellis 2001; Hartl et al. 2011). In the living cell there are also other factors that can increase the risk of protein aggregation, such as cellular stress, translocation across membranes, and the assembly of multiple domains (Ellis et al. 1991; Buchner 1996; Hartl 1996; Johnson et al. 1997; Nathan et al. 1997). In order for proteins to avoid the many obstacles that prevent proper folding and lead to aggregation within the cellular environment, cells employ a complex network of molecular chaperones (Ellis 1987; Young et al. 2004; Hartl et al.

2011). These molecular chaperones help proteins to avoid the danger of aggregation and aberrant folding within the cellular environment (Ellis 1987; Nathan et al. 1997; Young et al. 2004; Hartl et al. 2011). They also assist proteins in sustaining conformational flexibility, allowing them to shift from one conformation to another without being targeted for degradation (Nathan et al. 1997; Young et al. 2004; Hartl et al. 2011).

1.2 Molecular Chaperones

The term molecular chaperone was introduced by Ron Laskey and his colleagues while working on the formation of nucleosomes (Laskey et al. 1978; Ellis 1996). Laskey described a molecular chaperone as a protein required in the process of assembling nucleosome cores in egg extracts (Laskey et al. 1978; Ellis 1996). This term was later expanded by John Ellis in 1987, who proposed that 'molecular chaperone' could be used to describe a group of cellular proteins responsible for the correct folding and assembly of polypeptide chains into their oligomeric structures (Ellis 1987). When a functional three-dimensional conformation is not met, aggregation or degradation of certain proteins can give rise to disease states such as neurodegeneration, dementia, type 2 diabetes, cystic fibrosis, and cancer (Samuels et al. 1975; Breitner et al. 1986; Goate et al. 1991; Perutz 1999; Macario et al. 2000; Hull et al. 2004; Whitesell et al. 2005; Chiti et al. 2006; Morimoto 2008; Hahn 2009; Aguzzi et al. 2010; Hartl et al. 2011). Due to the large number of factors that contribute to protein aggregation and loss of function of cellular proteins, it is absolutely essential for a cell to contain a mechanism to create an environment that promotes proper folding (Nathan et al. 1997). Classical molecular chaperones prevent inappropriate intermolecular and intramolecular interactions,

stabilize folding intermediates, and are now known to be essential for the activity of a large range of signalling pathway molecules (Nathan et al. 1997; Lee et al. 2005; Buchanan et al. 2007). As steric information for polypeptide folding and oligomerization is not encoded by the chaperones themselves, it can be said that molecular chaperones are required for assistance in the proteins self-assembly, and aid in the acquisition of the proteins three dimensional structure by binding specific structural features (Ellis et al. 1991).

Many molecular chaperones are up-regulated during heat stress and are referred to as heat-shock proteins (Ellis et al. 1991). Heat shock proteins bind the interactive surfaces of proteins that are exposed during heat stress, and prevent these proteins from denaturing and forming inappropriate aggregates (Ellis et al. 1991). Like other molecular chaperones the heat-shock proteins also function within normal cells to serve a vital role at permissive temperatures (Lindquist et al. 1988). The heat shock proteins are grouped based on their molecular weight and can be divided into six groups known as the Hsp100 group, Hsp90 group, Hsp70 chaperones, Hsp60 chaperonins, Hsp40 DnaJ group, and the small Hsp or sHsp group (Macario et al. 2000; Lee et al. 2005). The Hsp100 family of heat shock proteins employ ATP to promote changes in folding and assembly of other proteins (Schirmer et al. 1996; Lee et al. 2005). The small Hsp or sHsp group is thought to bind several non-native proteins at a time, and cooperate with Hsp70 to prevent apoptosis (Jakob et al. 1993; Haslbeck 2002). The Hsp40 group bind substrate proteins and in many cases are responsible for the initial presentation of the unfolded substrates to Hsp70 to promote protein folding, transport, and degradation (Cheetham et al. 1998; Morano 2007). Possibly the most well studied family of heat shock proteins is the Hsp70 family whose members participate in a

partnership with other chaperones, such as Hsp90, in distinct functional networks (Voellmy et al. 1985; Tavaria et al. 1996; Morano 2007). The Hsp90 family members are essential heat shock proteins that possess an ATPase activity required for the activation, assembly, and folding of a specific set of 'client' proteins involved in signal transduction, transcriptional regulation, and cell cycle control (Panaretou et al. 1998; Pearl et al. 2000). Hsp90 exists within the cytosol of eukaryotes in two virtually identical isoforms known as constitutive and induced (Nathan et al. 1999; Cox et al. 2003; Hawle et al. 2006). The Hsp90 family also includes paralogues found within eukaryotic ER and mitochondria, however these members are less well understood and will not be discussed in this thesis (Maki et al. 1990; Song et al. 1995; Felts et al. 2000; Richter et al. 2007; Johnson et al. 2009).

1.3 Heat Shock Protein 90

Hsp90 is a highly conserved and abundant 90kDa molecular chaperone that plays a vital role in housekeeping functions, including protein trafficking, proliferation and apoptosis (Nathan et al. 1997; Pratt et al. 1997; Pratt et al. 2003; Retzlaff et al. 2009). It is one of the most abundant molecular chaperones accounting for 1-2% of all cellular proteins, even under normal conditions, and increasing up to 10-fold under physiological stress and within tumor cells (Nathan et al. 1997; Buchner 1999). Hsp90 naturally exists as an obligate homodimer made up of two identical monomers (Pratt et al. 1997; Scheibel et al. 1999; Hawle et al. 2006; Hainzl et al. 2009). As shown in Figure 1.1, each monomer consists of three domains; a C-terminal dimerization domain (Hsp90C), a middle domain (Hsp90M), and an N-terminal nucleotide binding ATPase domain (Hsp90N) (Prodromou et al. 1997; Scheibel et al. 1999; Richter et al. 2002). The

C-terminal domain of eukaryotic Hsp90 contains the main dimerization site for the Hsp90 homodimer (Weikl et al. 2000; Richter et al. 2002). Eukaryotic Hsp90C also contains a conserved pentapeptide sequence (MEEVD) that is the primary binding site for a specific set of co-chaperone proteins (Young et al. 1998). The middle domain binds both client proteins and co-chaperones with high affinity and is believed to control Hsp90's ability to discriminate between, and activate, different client proteins (Lotz et al. 2003; Meyer et al. 2003; Harst et al. 2005; Hawle et al. 2006). The middle domain of eukaryotic Hsp90 is connected to the N-terminal domain by a non-conserved charged middle linker region, made up of mostly charged amino acids, that is believed to provide flexibility to the Hsp90 protein (Louvion et al. 1996; Ali et al. 2006). The N-terminal domain of Hsp90 also contains a 30 amino acid residue lid segment involved in stabilization of N-domain association and ATP hydrolysis (Chadli et al. 2000; Prodromou et al. 2000; Ali et al. 2006).

The catalytic loop of Hsp90M and the lid segment of Hsp90N assist in setting up the catalytic apparatus of Hsp90 for ATP hydrolysis (Ali et al. 2006). Hsp90 is a member of the gyrase-Hsp90-histidine kinase-MutL (GHKL) family of homodimeric ATPases (Ban et al. 1999; Dutta et al. 2000). What is unique about these ATPases is the ability of their N-terminal domains to dimerize trapping ATP (Ban et al. 1998; Dutta et al. 2000; Bracher et al. 2006). Hsp90 function relies on its ability to bind ATP, when its N-terminal domains are dimerized, in any of its dynamic conformations, and to hydrolyze ATP which occurs only when Hsp90's N-termini come together in a closed conformation (Panaretou et al. 1998; Ali et al. 2006).

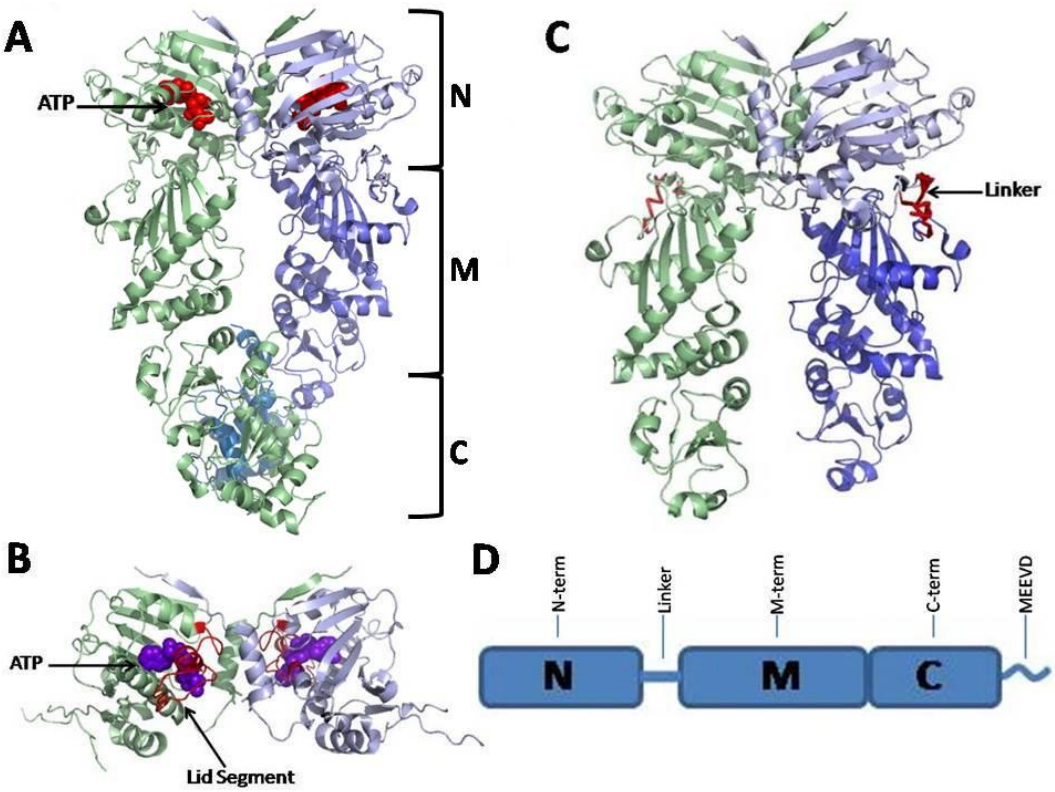

Figure 1.1: Hsp90 Crystal Structures. Crystal structures of the (A) full length yeast Hsp90, Hsp82p, in an ATP-bound state, (B) N-terminal domain, in an ATP bound state, with highlighted lid segment, and (C) Hsp90N and Hsp90M with highlighted charged linker region. (D) A simplified model of one Hsp90 monomer indicates the location of the N-terminal domain, conserved linker region, middle domain, C-terminal domain, and MEEVD conserved pentapeptide.

Hsp90 differs from classical chaperones as it is thought to bind specific secondary or tertiary structures of partially or fully folded proteins rather than binding to any protein purely by hydrophobic interactions like other chaperones (Pearl et al. 2006; Wayne et al. 2010). It is involved in assisting the maturation, maintenance, folding and conformational regulation of the unstable conformations of its client proteins (Xu et al. 1999; Pearl et al. 2000; Miyata 2005).

1.4 Hsp90 Interacts With a Specific Set of Client Proteins

There are many different types of Hsp90 client proteins that vary greatly in structure and function and range from transcription factors to ion channels to protein kinases to steroid receptors (Pratt et al. 1997; Xu et al. 1999; Miyata 2005; Echeverria et al. 2011). However, Hsp90 interacts with very specific structures found within the secondary or tertiary structure of its clients, therefore, simply being a transcription factor, ion channel, kinase, or steroid receptor does not imply the protein is necessarily an Hsp90 client (Nathan et al. 1995; Pratt et al. 1997; Pearl et al. 2000; Citri et al. 2006; Pearl et al. 2006; Echeverria et al. 2011). An up-to-date list of Hsp90 client proteins is available online thanks to Dr. Didier Picard (http://www.picard.ch/downloads/Hsp90interactors.pdf). The different types of Hsp90 client proteins also interact with Hsp90 in different ways (Pratt et al. 1997; Bracher et al. 2006; Pearl et al. 2006; Terasawa et al. 2006; Millson et al. 2008; Ran et al. 2008). In the case of steroid hormone receptors and transcription factors, client activation depends on a multichaperone machine including Hsp90 and Hsp70 (Bracher et al. 2006; Ran et al. 2008). Both Hsp90 and Hsp70's C-terminal domains contain a highly conserved pentapeptide sequence (MEEVD or IEEVD respectively) that serves as an anchor for

tetratricopeptide repeat (TPR) containing co-chaperone proteins (Young et al. 1998; Bracher et al. 2006; Millson et al. 2008). The upstream sequences of these peptides differ greatly between Hsp90 and Hsp70 (Scheufler et al. 2000; Pearl et al. 2006). Within yeast Hsp90 the peptide sequence is DDTSRMEEVD and in yeast Hsp70 the sequence is GSGPTIEEVD, therefore various TPR domains are capable of specifically binding one or the other of these peptide sequences (Scheufler et al. 2000; Pearl et al. 2006). The tetratricopeptide repeat (TPR) domain found in many Hsp90 co-chaperone proteins is a 34 amino acid motif that folds into a domain that binds to peptides within Hsp90 or Hsp70 via a carboxylate clamp (Lamb et al. 1995; Young et al. 1998; Scheufler et al. 2000). It is the co-chaperone proteins involved in the Hsp90 system that bind one or more of Hsp90's domains in an ATP dependant, sequential manner, that ultimately guide Hsp90 through its functional cycle (Obermann et al. 1998; Panaretou et al. 1998; Lotz et al. 2003).

1.5 Hsp90 Function is Regulated by a Group of Proteins Known as Co-chaperones

Co-chaperones are proteins that assist the function of chaperones (Caplan 2003). Co-chaperone interactions regulate the progression of Hsp90 through its conformationally dynamic, ATPase driven cycle, however the precise mechanism of Hsp90's chaperone activity still remains unclear (Lotz et al. 2003; Hawle et al. 2006). Figure 1.2 outlines a simplified version of the Hsp90 conformational chaperone cycle (Figure 1.2). The cycle involves the delivery of client proteins such as steroid hormone receptors to Hsp90 by Hsp70 via the TPR-containing co-chaperone **H**sp70-Hsp90 **O**rganizing **P**rotein, HOP or Sti1p in yeast, that functions to reversibly link Hsp70 and

Hsp90 together (Chen et al. 1998; Caplan 2003; Bracher et al. 2006; Johnson et al. 2007). Sti1p contains three TPR domains, TPR1, TPR2A, and TPR2B (Scheufler 2000; Millson 2008). TPR1 of Sti1p associates with Hsp70, while TPR2A of Sti1p associates with Hsp90 (Scheufler et al. 2000; Millson et al. 2008). This client binding occurs while Hsp90 is in the ADP-bound open state, and causes conformational changes in which Hsp90 conforms to an ATP bound closed state in association with another co-chaperone, p23 or Sba1p in yeast (Johnson et al. 1995; Ali et al. 2006). Another method of client delivery to Hsp90 involves client kinases in complex with the co-chaperone **C**ell-**D**ivision **C**ycle **37** homologue (Cdc37) (Roe et al. 2004; Terasawa et al. 2006; Millson et al. 2008). Cdc37 is thought to bind the N-terminal domain of Hsp90 at the ATP lid, and the N-lobe of protein kinases at the highly conserved glycine-rich loop (Roe et al. 2004; Terasawa et al. 2006; Millson et al. 2008). In the case of both steroid receptors and kinases, once the client protein has been activated, a third co chaperone, **A**ctivator of **H**sp90 **A**TPase 1 (Aha1), binds Hsp90 and stimulates ATP hydrolysis by Hsp90 which is thought to cause release of the client protein (Panaretou et al. 2002; Lotz et al. 2003; Meyer et al. 2004).

An important trait found in some co-chaperones is the ability to inhibit or stimulate Hsp90's essential ATPase activity (Caplan 2003; Gaiser et al. 2010). The only co-chaperone known to strongly stimulate Hsp90's ATPase activity is Aha1p, which is of significant importance as it is also known to be critical in a variety of disease pathways including cancer (Panaretou et al. 2002; Lotz et al. 2003; Hawle et al. 2006; Holmes et al. 2008; Gaiser et al. 2010). Significantly, Aha1p and other co-chaperones regulate Hsp90 function which is critical for maintaining the hyperactivation of client oncoproteins in cancer cells, making Hsp90 a good target for new cancer therapeutics (Whitesell et al. 2005; Ali et al. 2006; Hahn 2009).

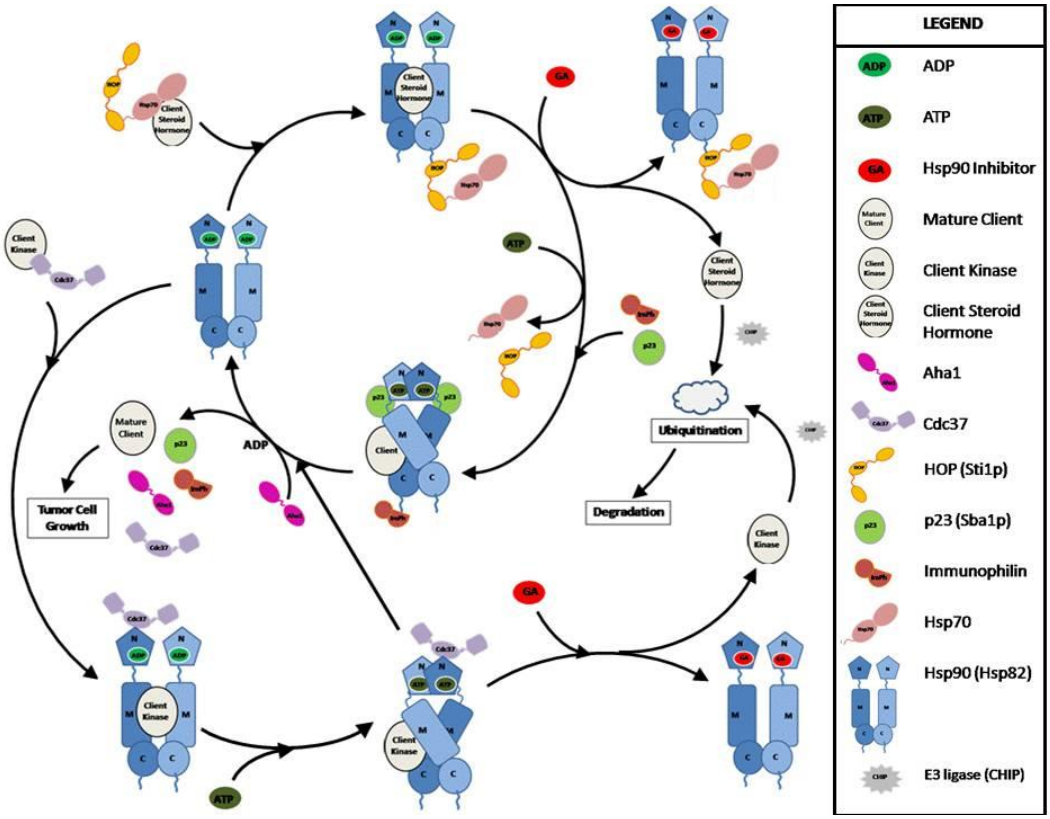

Figure 1.2: A simplified model of the Hsp90 cycle. The co-chaperone proteins, Hsp70 and HOP (Sti1p), aid in the binding of client steroid hormones to Hsp90, in an ADP-bound state. Upon binding ATP, Hsp70 and HOP (Sti1p) are released and the co-chaperones, p23 (Sba1p) and immunophilins (ImPh), bind Hsp90, preparing Hsp90 for ATP hydrolysis. The co-chaperone protein, Cdc37, aids in the binding of client kinases to Hsp90, in an ADP-bound state. Binding of ATP and N-terminal dimerization prepares this complex for ATP hydrolysis by Hsp90. The co-chaperone, Aha1 stimulates Hsp90 to hydrolyze ATP, therefore, releasing mature client proteins, many of which cause tumor cell growth. Hsp90-inhibitor drugs that bind the nucleotide pocket of Hsp90N, such as geldanamycin (GA), inhibit ATP binding and hydrolysis. This subsequently leads to ubiquitination of client proteins by the E3 ligase, CHIP, and thus targets clients for degradation.

1.6 Importance in Cancer

Hsp90 facilitates the activation of more than 200 client proteins, including many oncoproteins in cancer cells, and drives aberrant signalling (Pratt et al. 2003; Mimnaugh et al. 2006; Hahn 2009; Trepel et al. 2010). Hsp90 facilitates the hyperactivation of oncoproteins, enabling growth signalling and tumor cell proliferation (Pratt et al. 2003; Trepel et al. 2010; Centenera et al. 2012). Cancer cells depend on Hsp90 and are thus sensitive to Hsp90 inhibitors, which are capable of potentially targeting multiple signalling pathways implicated in the progression of cancer (Pratt et al. 2003; Trepel et al. 2010; Pimienta et al. 2011; Centenera et al. 2012). Hsp90 is a target for drug therapy in cancer clinical studies using Hsp90 inhibitor drugs such as Geldanamycin (GA), Radicicol, and NVP-AUY922 (Stebbins et al. 1997; Roe et al. 1999; Ge et al. 2006; Brough et al. 2008; Eccles et al. 2008; Hao et al. 2010). Majority of the Hsp90 inhibitors that are currently available bind the Hsp90 ATP binding pocket of the N-terminal domain as displayed in Figure 1.2 (Stebbins et al. 1997; Hao et al. 2010). These inhibitors block the processing of Hsp90 client proteins by preventing nucleotide from binding and thus preventing ATP hydrolysis, however these inhibitors are not simply non-hydrolyazable ATP analogues as they do not cause the same conformational changes as ATP or AMPPnP (Stebbins et al. 1997; Hao et al. 2010).

Geldanamycin has been considered an Hsp90 inhibitor since the mid-90's when it was determined to bind Hsp90 and disrupt its ability to chaperone the onco-kinase v-src (Whitesell et al. 1994; Stebbins et al. 1997). Although it has potent anti-tumor effects and disrupts ATP-dependent activation of a wide range of client proteins, it also has poor solubility and is highly hepatotoxic, therefore, new derivatives, 17AAG and 17DMAG, were introduced (Supko et al. 1995; Banerji et al. 2005; Ge et al. 2006). 17AAG

completed phase I clinical trials as a promising anti-cancer drug with lower toxicity than GA (Hostein et al. 2001; Munster et al. 2001; Ronnen et al. 2006; Hao et al. 2010). Unfortunately it too has poor solubility in water and thus must be dissolved in solutions, such as DMSO, that may interfere with the maximum tolerated dose of the drug due to the toxicity of these solutions (Egorin et al. 2001; Guo et al. 2005; Ge et al. 2006; Hao et al. 2010). The lower concentrations of 17AAG that must be administered do not have antitumor effects (Ronnen et al. 2006; Solit et al. 2008; Hao et al. 2010). 17DMAG is not only a more water soluble derivative of GA but is also a more potent antitumor Hsp90 inhibitor than 17AAG and is also being tested in clinical trials (Hollingshead et al. 2005; Hwang et al. 2006; Hao et al. 2010).

Radicicol was discovered in the 1950's as an anti-fungal antibiotic and is now recognized to destabilize Hsp90 client proteins by binding the nucleotide binding pocket with 50-fold greater affinity than GA (Schulte et al. 1998; Sharma et al. 1998; Soga et al. 1998; Ki et al. 2000; Hao et al. 2010). Like, Geldanamycin, Radicicol has potent anti-proliferative activity in human tumor cells *in vitro,* however, it too has poor solubility in water and must be dissolved in solutions that abolish its antitumor activity *in vivo* (Kwon et al. 1992; Kwon et al. 1997; Shiotsu et al. 2000; Hao et al. 2010).

NVP-AUY922 is currently the most potent, selective, small molecule Hsp90 inhibitor to be described (Eccles et al. 2008). While it inhibits proliferation *in vitro* in many human tumor cells, it also has powerful anti-tumor effects in a vast array of human tumor cells and potently inhibits tumor cell invasion and tumor vascularisation (Eccles et al. 2008). Research continues to provide new options that overcome the undesirable side effects of Hsp90 inhibitors for targeting Hsp90 for drug therapy in cancer in clinical settings (Pimienta et al. 2011). More recently Hsp90 cycle inhibitors

have been used to examine the effects of blocking co-chaperone binding to Hsp90 to prevent specific client processing by Hsp90 (Pimienta et al. 2011). Holmes *et al* revealed that the co-chaperone Aha1p is related to sensitivity of cells to Hsp90-inhibitors, as Aha1p was sustainably upregulated when cells were treated with the Hsp90 inhibitor 17AAG in a variety of human cancer cell lines, regardless of the basal levels of Aha1p (Holmes et al. 2008). Aha1p is also involved in the phosphorylation and activation of specific client kinases, and may increase the activity of other Hsp90 clients, many of which are involved in disease (Harst et al. 2005; Holmes et al. 2008). Aha1p is therefore important in studies involving Hsp90 and cancer clinical trials and could be a potential target for Hsp90 co-chaperone inhibitor drugs.

1.7 The Hsp90 Co-chaperones *AHA1* and *HCH1*, Similar Sequence, Different Function

It has only been roughly ten years since it was agreed upon that Hsp90 is an ATPase due to its almost undetectable levels of ATPase activity (Soti et al. 2003). The *in vitro* ATPase rate of human Hsp90 is around 1 ATP every 20 minutes while yeast Hsp90 is 20 times more active, yet still slow as only 1 ATP is turned over each minute (Panaretou et al. 1998; McLaughlin et al. 2002; Gaiser et al. 2010). Although Hsp90 is a very slow ATPase, the hydrolysis of ATP by Hsp90 is essential to sustain its function and is also essential to maintain viability in cells (Obermann et al. 1998; Panaretou et al. 1998; Richter et al. 2007). The co-chaperone Aha1p is involved in numerous substrate maturation pathways and binds both the middle and N-terminal domains of Hsp90 (Figure 1.3 (Panaretou et al. 2002; Lotz et al. 2003; Meyer et al. 2004; Harst et al. 2005; Hawle et al. 2006; Koulov et al. 2010)). It was initially thought to be the only co-

chaperone known to stimulate the ATPase rate of Hsp90, stimulating its low intrinsic ATPase activity by up to 10-fold (Panaretou et al. 2002; Lotz et al. 2003; Meyer et al. 2004; Hawle et al. 2006). Aha1p consists of an N-terminal and a C-terminal domain (Figure 1.3) and is thought to assist in client maturation by promoting Hsp90 N-terminal dimerization, ATP hydrolysis, and release of mature client proteins (Panaretou et al. 2002; Lotz et al. 2003; Meyer et al. 2004; Retzlaff et al. 2010). Aha1p plays a critical role in the maturation of Hsp90 clients, most importantly the oncogenic clients such as the kinase vsrc, thus making Aha1p of great importance when studying the mechanisms of the Hsp90 pathway and the maturation of its clients (Panaretou et al. 2002; Lotz et al. 2003; Holmes et al. 2008). Another Hsp90 co-chaperone, known as Hch1p, was identified in a screen as a protein that, when over-expressed, rescues an Hsp90 temperature-sensitive mutant, Hsp82p^{E381K}, in yeast (Nathan et al. 1999). Interestingly, *HCH1* shares 36.6% sequence identity and 50% similarity with the N-terminus of *AHA1* (*AHA1*$^{1-156}$) (Figure 1.4) and is believed to competitively bind the same position of Hsp90M (Figure 1.3), however, Hch1p only weakly stimulates Hsp90's ATPase activity (Panaretou et al. 2002; Lotz et al. 2003; Meyer et al. 2004). Hch1p is only found in lower eukaryotes, such as yeast, and is thus often overlooked in the Hsp90 field (Nathan et al. 1999; Lotz et al. 2003). It is, however, the obvious relationship to Aha1p and its ability to restore the growth of cells expressing temperature-sensitive Hsp90 mutants in a yeast model system that makes this co-chaperone of interest to us in our research (Nathan et al. 1999).

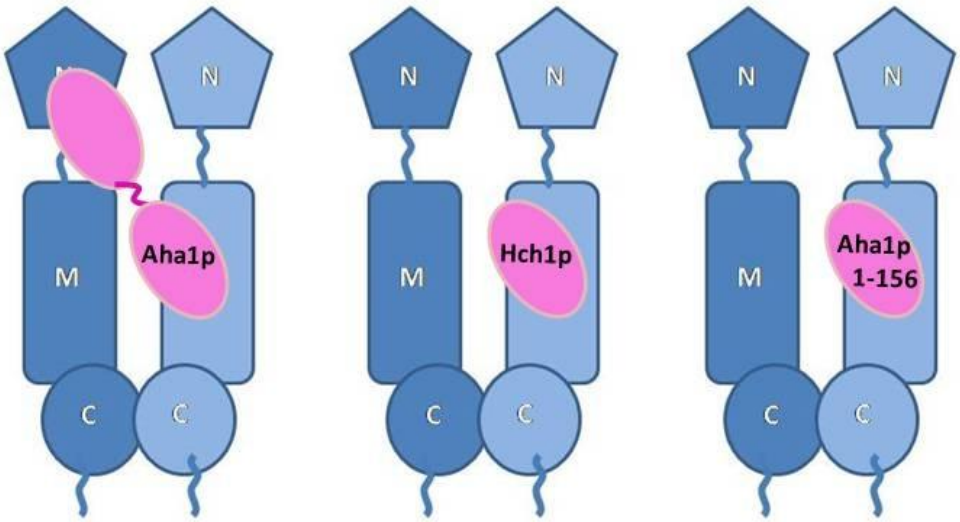

Figure 1.3: Schematic represents co-chaperone binding to Hsp82p. The co-chaperone Aha1p binds Hsp82p at the middle and N-terminal domains. The co-chaperone Hch1p binds Hsp82p at the middle domain. The N-terminal domain of Aha1p, Aha1p$^{1-156}$, binds Hsp82p at the middle domain.

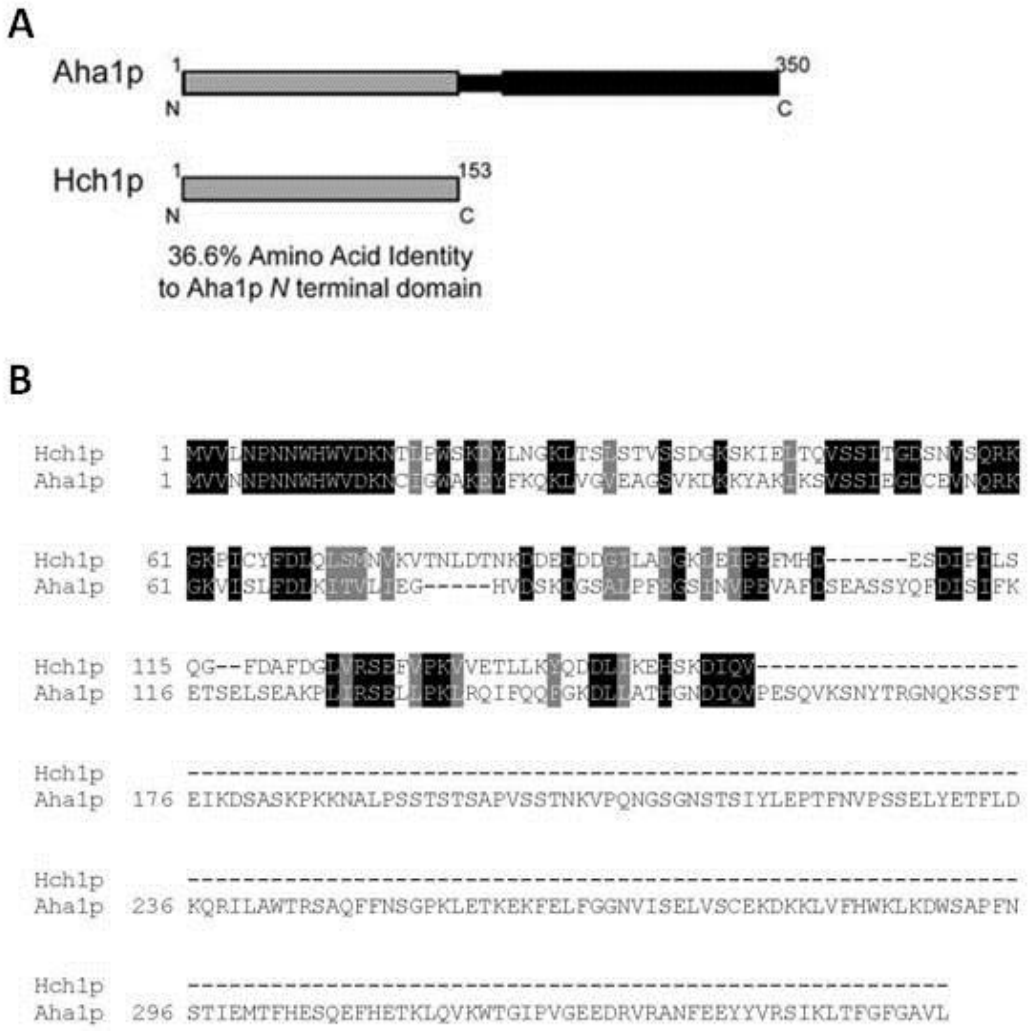

Figure 1.4: (A) Domain structures indicate the alignment of the co-chaperone Aha1p, 350 amino acids in length, and the co-chaperone Hch1p, 153 amino acid in length. (B) Sequence alignment of the co-chaperone Hch1p with the N-terminus of the co-chaperone Aha1p. Figure published by Armstrong *et al.* (Armstrong et al. 2012).

1.8 Yeast as a Model System

Hsp90 is expressed from prokaryotes to eukaryotes and is essential for eukaryotic cell survival (Nathan et al. 1999; Picard 2002). Hsp90 is present in eukaryotes in two isoforms in the cytosol, called the constitutive and induced isoforms (Csermely et al. 1998; Sreedhar et al. 2004). Within organisms these isoforms are practically identical and orthologues between organisms are highly conserved (Csermely et al. 1998; Millson et al. 2004; Sreedhar et al. 2004). Other components of the Hsp90 cycle are also maintained between various organisms such that components of the yeast and human Hsp90 systems are functionally interchangeable (Bohen 1998; Morano et al. 1998; Hao et al. 2010). The human Hsp90α and the inducible *Saccharomyces cerevisiae* Hsp90 gene, known as *HSP82,* share 60% amino acid identity and the relationship between Hsp90 and its client oncoproteins is maintained whether in human cells or yeast cells (Bohen 1998; Dollins et al. 2007; Lee et al. 2011). *S. cerevisiae* is therefore a useful model system in medical research surrounding the Hsp90 pathway in humans and in cancer, among other diseases (Bohen 1998; Morano et al. 1998; Nathan et al. 1999; Hawle et al. 2006; Hao et al. 2010). As Hsp90 is essential for cell viability, we used yeast strains containing temperature-sensitive (*ts*) Hsp90 mutants that grow normally at permissive temperatures but display growth defects at higher, restrictive temperatures (Nathan et al. 1995; Nathan et al. 1997; Nathan et al. 1999). Many of these Hsp90 *ts* mutants are not classical *ts* folding mutants (Nathan et al. 1995; Nathan et al. 1997; Nathan et al. 1999). Interestingly, while each of the strains displays a *ts* growth defect at restrictive temperatures, these mutant forms of Hsp82p do not lose activity at restrictive temperatures due to misfolding as classical *ts* mutants do (Nathan et al. 1995; Nathan et al. 1997; Nathan et al. 1999). These *ts* mutants of yeast Hsp90, each

containing a different point mutation, instead display temperature-sensitive defects due to impaired activity that prevents them from functioning at wild-type levels at high temperatures (Nathan et al. 1995; Nathan et al. 1997; Nathan et al. 1999). Specifically, each of these *ts* mutants displays a unique molecular impairment at a different step of the Hsp90 cycle, therefore, in order to help reveal key insights into the functions of the Hsp90 system *in vivo*, several *ts* mutants have been created (Nathan et al. 1995; Nathan et al. 1997; Nathan et al. 1999; Hawle et al. 2006).

1.9 The Hsp90 Temperature-sensitive Mutants, Hsp82p^{E381K}, Hsp82p^{A587T}, and Hsp82p^{G313S}

The eight *ts* mutants that we began our research with include Hsp82p^{T22I}, Hsp82p^{A41V}, Hsp82p^{G81S}, Hsp82p^{T101I}, Hsp82p^{G170D}, Hsp82p^{G313S}, Hsp82p^{E381K}, and Hsp82p^{A587T} (Nathan et al. 1995). Of these eight mutants, only Hsp82p^{G170D} has been shown to be a classical *ts* mutant (Nathan et al. 1995; Nathan et al. 1997; Nathan et al. 1999). Classical *ts* mutants lose activity *in vivo* because they misfold at restrictive temperatures (Nathan et al. 1995; Nathan et al. 1997; Nathan et al. 1999). In contrast, the other seven Hsp90 *ts* mutants are partial loss-of-function mutants and are not misfolded at high temperatures (Nathan et al. 1995). They lose activity due to an inability of mutant Hsp90 to deal with the increased demand for Hsp90 function when cells are under stress (Nathan et al. 1995).

Previous experiments have shown that cells expressing Hsp82p^{E381K} as their sole source of Hsp90 have severe growth defects causing them to grow at only half the rate of wild-type cells at 25°C (Nathan et al. 1999). When temperatures are increased to 34°C cells expressing Hsp82p^{E381K} grow even more slowly and above 35°C these cells are no

longer viable (Nathan et al. 1995). Hsp82p^{E381K} mutant cells containing a wild-type Hsp90 plasmid do not have temperature-sensitive growth phenotypes from 25-37°C, thus Hsp82p^{E381K} is a recessive mutation (Nathan et al. 1995). Over-expression of the multicopy suppressor *HCH1* is capable of restoring wild-type levels of growth in cells expressing Hsp82p^{E381K} as their sole source of Hsp90 up to 37°C (Nathan et al. 1999). Interestingly, over-expression of *HCH1* also caused reduced levels of growth in the mutants Hsp82p^{G313S} and Hsp82p^{A587T} (Nathan et al. 1999). As we are attempting to determine the function of Hch1p and its relationship with its homologue, Aha1p, we chose to specifically study the Hsp90 mutants Hsp82p^{G313S}, Hsp82p^{E381K}, and Hsp82p^{A587T} (Figure 1.5).

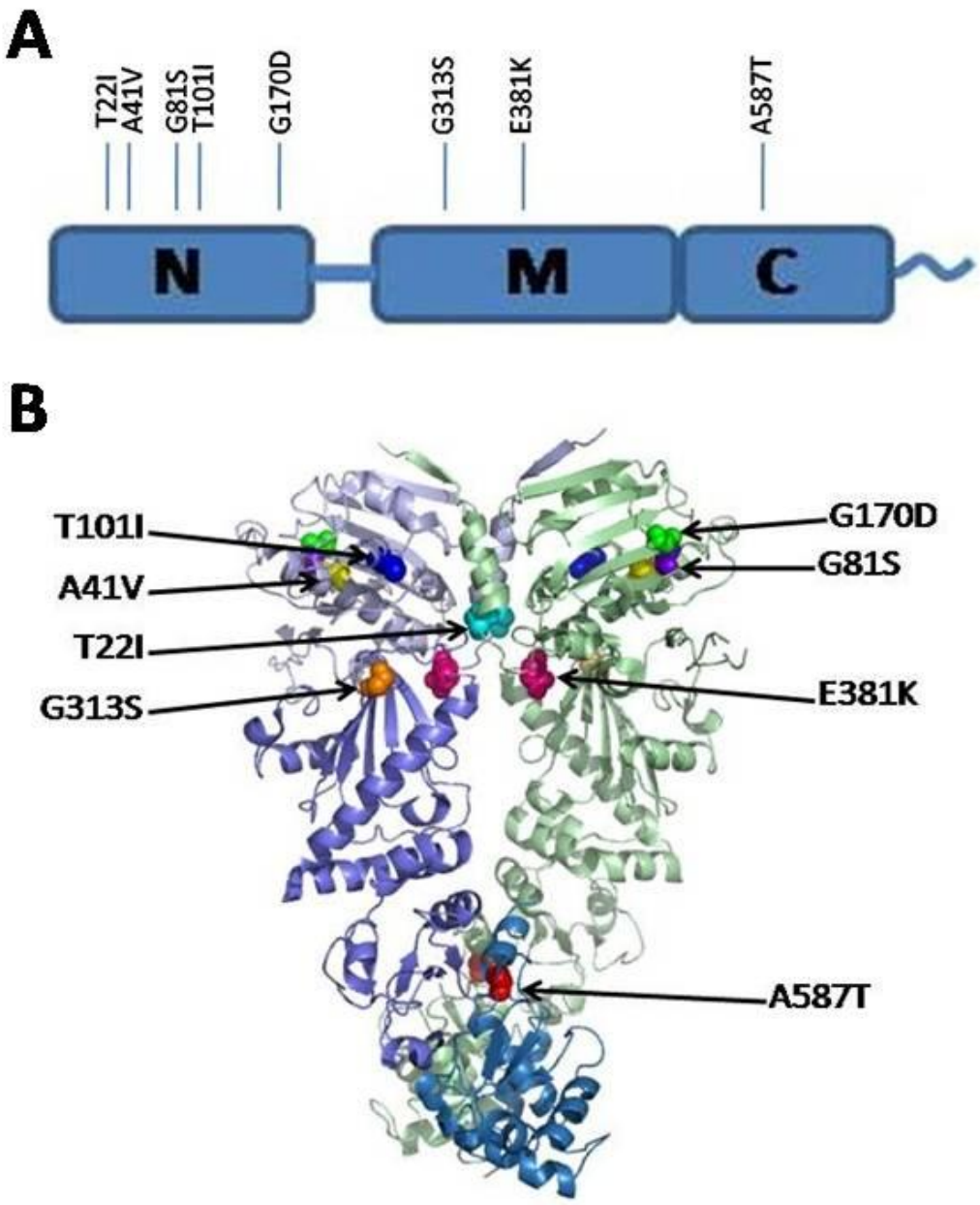

Figure 1.5: (A) Location of the eight mutations that result in temperature-sensitive growth in yeast; N denotes Hsp82pN, M denotes Hsp82pM, and C denotes Hsp82pC. (B) Location of the eight mutations in the 3-dimensional structure of the Hsp82p dimer.

1.10 Rational for Thesis

There is much that remains to be elucidated about the Hsp90 cycle and its involvement in disease states. Importantly, Hsp90 is proving to be a key target in clinical studies due to its importance in regulating disease related client proteins such as the onco-kinase, vsrc, in cancer (Panaretou et al. 2002; Pratt et al. 2003)P(Trepel et al. 2010; Centenera et al. 2012). Hsp90s dynamic, ATPase driven, cycle is regulated by interactions with co-chaperone proteins (Lotz et al. 2003; Hawle et al. 2006). One specific co-chaperone, Aha1p, is of interest in clinical studies due to its involvement in regulating disease related Hsp90 clients (Harst et al. 2005; Holmes et al. 2008). Aha1p not only stimulates Hsp90's essential ATPase activity, it has also been shown to be sustainably up-regulated in a variety of human cancer cell lines following treatment with Hsp90 inhibitor drugs, indicating a potential therapeutic approach to increasing sensitivity to Hsp90-inhibitors by depleting Aha1p (Whitesell et al. 2005; Ali et al. 2006; Holmes et al. 2008; Hahn 2009). Interestingly, over-expression of the co-chaperone Hch1p, homologous to the N-terminal domain of Aha1p, has been shown to recover growth of yeast expressing the Hsp90 mutant, Hsp82p^{E381K}, at high temperatures (Nathan et al. 1999). We hypothesized that as Hch1p and Aha1p are considered homologous, deletion of *HCH1* would have the same effect as deletion of *AHA1*.

To test our hypothesis, we obtained a series of yeast strains from Dr. Susan Lindquist, each with the endogenous *HSC82* and *HSP82* genes deleted but containing either the wild-type or mutant form of *HSP82* integrated at the TRP1 locus of the chromosome. These strains are detailed in Table 2.6 and include the wild-type, ip82, and eight *ts* mutant yeast strains; ipT22I, ipA41V, ipG81S, ipT101I, ipG170D, ipG313S, ipE381K, and ipA587T (Dr. Susan Lindquist, MIT; (Nathan et al. 1995)). We determined

that the *in vivo* impairments of yeast expressing Hsp82p^{G313S} or Hsp82p^{A587T} are exacerbated by the co-chaperone Hch1p, but not its homologue Aha1p (Armstrong et al. 2012). While deletion of *HCH1* alleviates temperature-sensitive impairments in cells expressing Hsp82p^{G313S} or Hsp82p^{A587T}, it is lethal in cells expressing Hsp82p^{E381K} (Armstrong et al. 2012). Strains expressing Hsp82p^{G313S} or Hsp82p^{A587T} are also hypersensitive to Hsp90 inhibitors and we were able to demonstrate that over-expression of *HCH1* confers hypersensitivity to Hsp90 inhibitors in yeast expressing wild-type Hsp82p or Hsp82p^{A587T} (Armstrong et al. 2012).

We believe that the observed temperature-sensitive phenotype of yeast expressing the Hsp90 mutant Hsp82p^{A587T}, Hsp82p^{G313S}, or Hsp82p^{E381K}, is due to an inability of mutant Hsp90 to deal with an increase in substrate burden on the Hsp90 system, and that the capacity for these mutants to process substrates under conditions of high burden is altered by Hch1p, but not its homologue Aha1p.

Table 1.1: Phenotypic characteristics of co-chaperone knock-out Hsp82p mutant strains observed in growth assays

		Yeast Strain								
°C	**Construct**	**ip82**	**iT22I**	**iA41V**	**iG81S**	**iT101I**	**iG170D**	**ipG313S**	**iE381K**	**ipA587T**
RT	URA plasmid	P/L	nd	nd	P/L	P/L	P/L	P/L	P/L	P/L
	Δhch1	P/L	nd	nd	P/L	P/L	P/L	P/L	nd	P/L
	Δaha1	P/L	nd	nd	P/L	P/L	P/L	P/L	P/L	P/L
28	URA plasmid	P/L	nd	nd	P/L	P/L	P/L	P/L	P/M	P/L
	Δhch1	P/L	nd	nd	P/L	P/L	P/L	P/L	nd	P/L
	Δaha1	P/L	nd	nd	P/L	P/L	P/L	P/L	P/M	P/L
34.5	URA plasmid	P/L	nd	nd	P/L	P/L	P/L	—/N	P/M	P/L
	Δhch1	P/L	nd	nd	P/L	P/L	P/L	W/S	nd	P/L
	Δaha1	P/L	nd	nd	P/L	P/L	P/L	—/N	P/M	P/L
37	URA plasmid	P/L	nd	nd	W/M	W/L	—/N	—/N	W/S	—/N
	Δhch1	P/L	nd	nd	W/M	W/L	—/N	W/L	nd	W/L
	Δaha1	P/L	nd	nd	W/M	W/L	—/N	—/N	W/S	W/M

* Phenotype includes colour and amount of growth of colonies observed in plated growth assays

** 'P' and 'W' describe pink and white colony colours; 'L', 'M', 'S', 'N' describe large, medium, small and no growth; 'nd' describes experiments that were not done in initial screenings

CHAPTER TWO

Materials and Methods

CHAPTER TWO: MATERIALS AND METHODS

2.1 Materials

2.1.1 Reagents

Reagents listed below were used according to procedures outlined by the Environmental Health and Safety of the University of Alberta and Workplace Hazardous Materials Information System (WHIMIS).

Table 2.1: Chemicals and reagents

Reagent	Supplier
ß-Mercaptoethanol	BioShop Canada Inc.
17AAG	LC Laboratories
Acetic acid; glacial	Fisher Scientific Canada
Acetone (80%)	Fisher Scientific Canada
Acrylamide/bis (30%; 29:1)	BioRad Laboratories
Agar	Fisher Scientific Canada
Agarose (UltraPure™)	Fisher Scientific Canada
Amino acids	MP Biomedicals, LLC
Ammonium sulphate	Fisher Scientific Canada
Ampicillin	CALBIOCHEM
APS (Ammonium Persulfate)	Life Technologies Inc.
Bacto™-Peptone	Becton, Dickinson and Company (BD)
Bacto™-Yeast Extract	Becton, Dickinson and Company (BD)
BSA (Bovine serum albumin)	Hoffmann-La Roche Ltd.

Butanol	Fisher Scientific Canada
Complete, EDTA-free protease inhibitor cocktail tablets	Hoffmann-La Roche Ltd.
Coomassie Blue Stain	BioRad Laboratories
DPBS (Delbecco's phosphate buffered saline)	Mediatech Inc.
Dextrose	Fisher Scientific Canada
Difco™ Yeast Nitrogen Base	Becton, Dickinson and Company (BD)
D-Galactose	Fisher Scientific Canada
DTT (dithiothreitol)	Hoffmann-La Roche Ltd.
DMSO (dimethyl sulfoxide)	Sigma-Aldrich Co. LLC.
dNTP (deoxyribonucleotide triphosphate)	New England BioLabs
EDTA (ethylenediamine-tetraacetic acid)	Fisher Scientific Canada
Ethanol	Commercial Alcohols
Fermentas GeneRuler 1 kb DNA Ladder	Fermentas - Thermo Fisher Scientific Inc
Fermentas PageRuler™ Protein Ladder Plus	Fermentas - Thermo Fisher Scientific Inc.
Geldanamycin	LC Laboratories
Geneticin (G418)	Life Technologies Inc.
Glycerol	Fisher Scientific Canada
Glycine	MP Biomedicals, LLC
HALT Protease Inhibitor	Thermo Fisher Scientific Inc.
Hydrochloric acid	Fisher Scientific Canada
Hydrogen Peroxide	Fisher Scientific Canada
Isopropanol	Fisher Scientific Canada

KCl (Potassium chloride)	Fisher Scientific Canada
LB Broth, Miller	Fisher Scientific Canada
Lithium Acetate	Acros Organics
Luminal (3-Aminophthalhydrazide)	Sigma-Aldrich Co. LLC.
Methanol	Fisher Scientific Canada
MgCl (Magnesium chloride)	Fisher Scientific Canada
NaCl (Sodium chloride)	Fisher Scientific Canada
NEB Buffer	New England BioLabs
Nitrocellulose membrane	BioRad Laboratories
NVP-AUY922	Chemie Tek
p-Coumaric Acid	Sigma-Aldrich Co. LLC.
PCR Primers	Integrated Device Technology
PEG3550	Fisher Scientific Canada
PfuTurbo DNA polymerase	Stratagene
Ponceau stain	MP Biomedicals, LLC
PMSF (Phenylmthylsulfonyl Fluoride)	Fisher Scientific Canada
Raffinose	MP Biomedicals, LLC
Restriction endonucleases	New England BioLabs
SDS (sodium dodecyl sulphate)	Fisher Scientific Canada
Skim milk powder	Carnation
Sodium Chloride	Fisher Scientific Canada
Sodium hydroxide	Fisher Scientific Canada
Sorbitol	Fisher Scientific Canada
SYBR Safe DNA gel stain	Invitrogen - Life Technologies

	Corporation.
T4 DNA ligase	New England BioLabs
TCA (Trichloroacetic Acid)	Fisher Scientific Canada
TEMED (tetramethylethylenediamine)	Fisher Scientific Canada
TopTaq DNA Polymerase	QIAGEN
Tris (tris-(hydrocymethyl)aminomethane)	Fisher Scientific Canada
Triton X-100	Fisher Scientific Canada
Tween 20	Fisher Scientific Canada

Table 2.2: Commercial Kits

Kit	Supplier
ECL Plus Western Blotting Detection System	GE Healthcare
QIAGEN Plasmid Midi kit	QIAGEN
QIAprep spin miniprep kit	QIAGEN
QIAquick gel extraction kit	QIAGEN
QIAGEN PCR Purification kit	QIAGEN

2.1.2 Commonly Used Buffers

Table 2.3: Commonly Used Buffers and Medias

Buffer/Medium Name	Buffer/Medium Components
Coomassie Stain (0.1%)	2g Coomassie Blue Stain 2L Destain
Destain	2.105L 95% Ethanol 2.0L Glacial Acetic Acid 15.895L ddH_2O
ECL Solution #1-Luminol	2.5mM luminal (3-Aminophthalhydrazide) 0.45mM p-Coumaric Acid 0.1M Tris Base pH 8.8

ECL Solution #2-H_2O_2	0.02% Hydrogen Peroxide 0.1M Tris pH 8.8
10X Electrode	600g Tris Base 2880g Glycine 200g SDS Up to 20L ddH_2O
LB Medium	25g LB Broth, Miller 1L ddH_2O (Add 20g Agar for plates)
LiAcTE	100mM Lithium Acetate 10mM Tris pH 7.2 1mM EDTA
Lysis Buffer	370µl 6M NaOH 75µl ß-Mercaptoethanol 56µl 200mM PMSF 500µl ddH_2O
PEG	LiAcTE 40% PEG3550
Ponceau Stain	1g Ponceau S 50mL Acetic Acid Up to 1L ddH_2O
4X Running Gel Buffer	363.4g Tris Base 1600mL ddH_2O pH 8.8 (with HCl or NaOH) 8g SDS (Sodium dodecyl sulphate) Up to 2L ddH_2O
Synthetic Complete Yeast Medium	1.7g Yeast Nitrogen Base 5g Ammonium Sulphate <u>(Add necessary amino acids)</u> 6mL L-His, L-Leu 10mL L-Arg, L-Met, L-Iso, L-Lys, L-Asp, L-Val, L-Ser 10mL Adenine Sulphate 10mL L-Phe, L-Gly, L-Thr 10mL L-Ura 2mL L-Trp 25mL Tyrosine <u>(Add necessary sugars)</u>

	50mL 40% Glucose 100mL 20% Galactose 100mL 20% Raffinose (Add 20g Agar for plates)
SDS-PAGE sample buffer (6X)	30% Glycerol 120mM Tris pH 7.0 6% SDS 0.6% Bromophenol Blue
SDS-PAGE sample buffer (2X)	90µl SDS-PAGE sample buffer (6X) 30µl DTT 200µl ddH_2O 10µl HALT protease inhibitor
Stacking Gel Buffer	33.92g Tris Base 1800mL ddH_2O pH6.8 2.2g SDS Up to 2L ddH_2O
50X TAE	242g Tris Base 57.1mL Glacial Acetic Acid 100mL 0.5M EDTA pH 8.0 (with acetic acid)
TBS (Tris-buffered saline)	320g NaCl 8g KCl 120g TrisBase pH 8.0 4L ddH_20
10X Western Transfer Buffer	605g Tris Base 2880g Glycine Up to 20L ddH_2O
YPD Medium	10g Yeast Extract 20g Peptone 1L ddH_2O (Add 20g Agar for plates)

2.1.3 Oligonucleotides

The following oligonucleotides were used in a polymerase chain reaction to target specific genes, add tags, and create specific mutations, as indicated in Table 2.5.

Table 2.4: Primers used in molecular cloning

Primer Number	Primer Name	Sequence
0001	sensePCRKOAHA1	tcttattcttaatcgtttatagtagcaacaatatatcaataagattgtactgagagtgcac
0002	antisensePCRKOAHA1	atttacgcatacttttattgaaacatgagaacaatatatcctgtgcggtatttcacaccg
0003	sensePCRKOHCH1	ttgaaacgatcggaaagttacaacacatttacgataaaatagattgtactgagagtgcac
0004	antisensePCRKOHCH1	tctatctatgcaacgctccccttttcgttacatgaacacactgtgcggtatttcacaccg
0009	senseScHsp82BamHI	gagagaggatccatggctagtgaaacttttgaatttcaagc
0010	senseScHsp82mycBamHI	gagagaggatccatggaacaaaaattgatttctgaagaagatttgatggctagtgaaacttttgaatttcaagc
0011	antisenseScHsp82XhoI	gagagactcgagtcactaatctacctcttccatttcg
0012	antisenseScHsp82mycXhoI	gagagactcgagtcactacaaatcttcttcagaaatcaatttttgttcatctacctcttccatttcg
0013	senseScAha1BamHI	gagagaggatccatggtcgtgaataacccaaataactg
0014	senseScAha1mycBamHI	gagagaggatccatggaacaaaaattgatttctgaagaagatttgatggtcgtgaataacccaaataactg
0016	antisenseScAha1mycXhoI	gagagactcgagtcactacaaatcttcttcagaaatcaatttttgttctaatacggcaccaaagccgaatgtc
0017	senseScHch1BamHI	gagagaggatccatggttgtcttgaatccaaataactg
0018	senseScHch1mycBamHI	gagagaggatccatggaacaaaaattgatttctgaagaagatttgatggttgtcttgaatccaaataactg
0020	antisenseScHch1mycXhoI	gagagactcgagtcactacaaatcttcttcagaaatcaatttttgttcaacttgtatatcctttgagtg
0060	senseScHch1BglIINcoI	gagagaagatctccatgggccatcatcatcatcatcatatggttgtcttg

0073	antisenseHCH1SacI	gagagagagctctcactaaacttgtatatcctttgagtgttc
0075	sense BglIImycNdeIScHch1	gagagaagatctatggaacaaaaattgatttctgaagaagatttgcatatggttgtcttg
0076	antisenseAHA1NotI	gagagagcggccgctcactataatacggcaccaaagccg
0077	senseBglIINdeIScHCH1	gagagaagatctcatatggttgtcttgaatccaaataactg
0078	antisenseScHCH1XhoIBamHI	gagagactcgagggatcctcactaaacttgtatatccttttgagtg
0090	antisenseHch1mycSacI	gagagagagctctcactacaaatcttcttcagaaatcaatttttgttccataacttgtatatcctttgagtgttc
0099	sQCHsp82T587AwtAfeI	cagaactggtcaatttggttggagcgctaacatggaaagaatcatgaaggc
0100	aQCHsp82T587AwtAfeI	gccttcatgattctttccatgttagcgctccaaccaaattgaccagttctg
0113	sQCHsp82G313SMfeI	gacccattgtacgttaagcatttctccgttgaatctcaattagaatttagagctatcttattcattccaaagagagcacc
0114	aQCHsp82G313SMfeI	ggtgctctctttggaatgaataagatagctctaaattctaattgagattcaacggagaaatgcttaacgtacaatgggtc
0123	sQCHsp82E381KXbaI	gactctgaggatttaccattgaatttgtctagaaaaatgttacaacaaaataagatcatg
0124	aQCHsp82E381KXbaI	catgatcttattttgttgtaacatttttctagacaaattcaatggtaaatcctcagagtc
0126	antisensemycAHA1NotI	gagagagcggccgctcactacaaatcttcttcagaaatcaatttttgttccattaatacggcaccaaagccg
0193	antisenseAHA1Nterm156mycNotI	gagagagcggccgctcactacaaatcttcttcagaaatcaatttttgttccatcacctgaatgtcattaccatgggtggccagc
0289	sQCHsp82V391E	gttacaacaaaataagatcatgaaggagattagaaagaacattgtcaaaaag

0290	aQCHsp82V391E	ctttttgacaatgttctttctaatctccttcatgatcttattttgttgtaac
0467	schimerantermhch1aha1	ctcaaaggatatacaagttcccgaatctcaggtg
0468	achimerantermhch1aha1	cacctgagattcgggaacttgtatatccctttgag
0493	sQCVLHchtoGPAha	cagttgtcgatgaacgtcaaaggacacgtggactctaaggacggatcggcattgccagcggacgggaaactagaaattccag
0494	aQCVLHchtoGPAha	ctggaatttctagtttcccgtccgctggcaatgccgatccgtccttagagtccacgtgtcctttgacgttcatcgacaactg
0495	sQCGPAhatoVLHch	gaaaatcacggtgttaatagaggtgacaaatttggatactaataaggacgacgaggatgatgacggcatactttcgagggtagcattaacgttcctg
0496	aQCGPAhatoVLHch	caggaacgttaatgctaccctcgaaaagtatgccgtcatcatcctcgtcgtccttattagtatccaaatttgtcacctctattaacaccgtgattttc
0497	sA12H	gaaggtgattgtgaagttaatcaaaggaagggcaagccg
0498	aA12H	cggcttgcccttcctttgattaacttcacaatcaccttc
0499	sH12A	caccggtgactccaacgtatctcagcgtaaggggaaggttatatc
0500	aH12A	gatataaccttccccttacgctgagatacgttggagtcaccggtg
0501	sA23H	ctaaggacggatcggcattgccagcggacgggaaactag
0502	aA23H	ctagtttcccgtccgctggcaatgccgatccgtccttag
0503	sH23A	cgaggatgatgacggcatacttttcgagggtagcattaacgttcctg
0504	aH23A	caggaacgttaatgctaccctcgaaaagtatgccgtcatcatcctcg

0507	antisenseScAha1Nterm156 mycXho1	gagagactcgagtcactacaaatcttcttcagaaatca atttttgttccacctgaatgtcattaccatgggtggcca gc
0508	sQCAha1V98toF109intoHc h1	cgggaaactagaaattccagaggttgccttcgatagc gaggcctcaagctatcaatttgatattccgattttgtcg caagggtttg
0509	aQCAha1V98toF109intoHc h1	caaacccttgcgacaaaatcggaatatcaaattgata gcttgaggcctcgctatcgaaggcaacctctggaattt ctagtttcccg
0510	sQCHch1F103toS108intoAh a1	cgagggtagcattaacgttcctgaatttatgcatgacg agtctgacatatctatatttaaggagac
0511	aQCHch1F103toS108intoA ha1	gtctccttaaatatagatatgtcagactcgtcatgcata aattcaggaacgttaatgctaccctcg
0512	sQCAha1F114toP125intoHc h1	gacgagtctgatattccgatttttaaggagactagcga attgagtgaagccaagccattggttaggtctgaatttg tccctaaag
0513	aQCAha1F114toP125intoH ch1	ctttagggacaaattcagacctaaccaatggcttggct tcactcaattcgctagtctccttaaaaatcggaatatca gactcgtc
0514	sQCHch1L113toG122intoA ha1	gctatcaatttgacatatctatattgtcgcaagggtttg atgcttttgatggactaattagatccgagttattgccca ag
0515	aQCHch1L113toG122intoA ha1	cttgggcaataactcggatctaattagtccatcaaaag catcaaacccttgcgacaatatagatatgtcaaattga tagc

2.1.4 Plasmid Vectors

Plasmid vectors created are listed in Table 2.5 indicating the selectable markers and cut sites integrated into the plasmids. Yeast strains available for integration of plasmid vectors are listed in Table 2.6 including the strains name, the specific form of Hsp82p expressed, the specific site of integration, and any specific genes that were deleted.

Table 2.5: Plasmid Vectors

Plasmid name	Selection	Primer/Enzymes	Derived from
pET11dHisHsp82	Amp^r, His_6		
pET11dHisA587T	Amp^r, His_6	0099 + 0100	pET11dHisHsp82
pET11dHisG313S	Amp^r, His_6	0113 + 0114	pET11dHisHsp82
pET11dHisE381K	Amp^r, His_6	0123 + 0124	pET11dHisHsp82
pET11dHisV391E	Amp^r, His_6	0289 + 0290	pET11dHisHsp82
pET11dHisV391E-G313S	Amp^r, His_6	0289 + 0290	pET11dHisG313S
pET11dHisV391E-A587T	Amp^r, His_6	0289 + 0290	pET11dHisA587T
pRS404GPD NTHCH1	TRP1	0077 + 0078	pET11dHis mycHCH1
pRS404GPD mycHCH1	TRP1, myc	0075 + 0078	pET11dHis mycHCH1
pRS404GPD HisHCH1	TRP1, His_6	0060 + 0078	pET11dHis mycHCH1
p404GPD NTHsp82	TRP1	Nde1 + BamHI	pRS404GPD NTHCH1
p404GPD HisHsp82	TRP1, His_6	Nde1 + BamHI	pRS404GPD HisHCH1
p404GPD NTG313S	TRP1	Nde1 + BamHI	pRS404GPD NTHCH1
p404GPD HisG313S	TRP1, His_6	Nde1 + BamHI	pRS404GPD HisHCH1
p404GPD NTA587T	TRP1	Nde1 + BamHI	pRS404GPD NTHCH1
p404GPD HisA587T	TRP1, His_6	Nde1 + BamHI	pRS404GPD HisHCH1
p404GPD NTE381K	TRP1	Nde1 + BamHI	pRS404GPD NTHCH1
p404GPD HisE381K	TRP1, His_6	Nde1 + BamHI	pRS404GPD HisHCH1
p404GPDNTV391E	TRP1	Nde1 + BamHI	pRS404GPD NTHCH1
p404GPDHisV391E	TRP1, His_6	Nde1 + BamHI	pRS404GPD HisHCH1
p404GPDNTV391E-G313S	TRP1	Nde1 + BamHI	pRS404GPD NTHCH1

p404GPDHisV391E-G313S	TRP1, His_6	Nde1 + BamHI	pRS404GPD HisHCH1
p404GPDNTV391E-A587T	TRP1	Nde1 + BamHI	pRS404GPD NTHCH1
p404GPDHisV391E-A587T	TRP1, His_6	Nde1 + BamHI	pRS404GPD HisHCH1
pRS426854 vc	GAL, URA3	Nde1 + BamHI	
pRS426854 NTHCH1	GAL, URA3	0017 + 0073	pRS426854
pRS426854 mycHCH1	GAL, URA3, myc	0018 + 0073	pRS426854
pRS426854 HCH1myc	GAL, URA3, myc	0017 + 0090	pRS426854
pRS426854 NTAHA1	GAL, URA3	0013 + 0076	pRS426854
pRS426854 mycAHA1	GAL, URA3, myc	0014 + 0076	pRS426854
pRS426854 AHA1myc	GAL, URA3, myc	0013 + 0126	pRS426854
pRS426854 $AHA1^{1-156}$myc	GAL, URA3, myc	0013 + 0193	pRS426854
p41KanTEF	GEN	KpnI + SacI	p424TEF into p41Kan
p41KanTEF HCH1myc	GEN, myc	0017 + 0020	p41KanTEF
p41KanTEF AHA1myc	GEN, myc	0013 + 0016	p41KanTEF
p41KanTEF $AHA1^{1-156}$myc	GEN, myc	0013 + 0507	p41KanTEF

Table 2.6: Yeast strains

Strain	Hsp82p expressed	Site of integration	Deletions	Notes
ip82	wt	HIS3		MIT
ipT22I	T22I	HIS3		MIT
ipA41V	A41V	HIS3		MIT
ipG81S	G81S	HIS3		MIT

ipT101I	T101I	HIS3		MIT
ipG170D	G170D	HIS3		MIT
ipG313S	G313S	HIS3		MIT
ipE381K	E381K	HIS3		MIT
ipA587T	A587T	HIS3		MIT
i(T/H)* Hsp82p	wt	TRP1/HIS3		Shuffling
i(T/H)* Hsp82p^{G313S}	G313S	TRP1/HIS3		Shuffling
i(T/H)* Hsp82p^{E381K}	E381K	TRP1/HIS3		Shuffling
i(T/H)* Hsp82p^{A587T}	A587T	TRP1/HIS3		Shuffling
i(T/H)* His Hsp82p	6xHisHsp82	TRP1/HIS3		Shuffling
i(T/H)* His Hsp82p^{G313S}	6xHisG313S	TRP1/HIS3		Shuffling
i(T/H)* His Hsp82p^{E381K}	6xHisE381K	TRP1/HIS3		Shuffling
i(T/H)* His Hsp82p^{A587T}	6xHisA587T	TRP1/HIS3		Shuffling
iT HisHsp82p^{V391E}	6xHisV391E	TRP1		Shuffling
iT HisHsp82p$^{G313S-V391E}$	6xHisG313S-V391E	TRP1		Shuffling
iT HisHsp82p$^{A587T-V391E}$	6xHisA587T-V391E	TRP1		Shuffling
i(T/H)* Hsp82p rdm	wt	TRP1/HIS3		Shuffling
i(T/H)* Hsp82p^{G313S} rdm	G313S	TRP1/HIS3		Shuffling
i(T/H)* Hsp82p^{E381K} rdm	E381K	TRP1/HIS3		Shuffling
i(T/H)* Hsp82p^{A587T} rdm	A587T	TRP1/HIS3		Shuffling
i(T/H)* His Hsp82p rdm	6xHisHsp82	TRP1/HIS3		Shuffling
i(T/H)* His Hsp82p^{G313S}	6xHisG313S	TRP1/HIS3		Shuffling

rdm				
i(T/H)* His Hsp82p^{E381K} rdm	6xHisE381K	TRP1/HIS3		Shuffling
i(T/H)* His Hsp82p^{A587T} rdm	6xHisA587T	TRP1/HIS3		Shuffling
ip82Δhch1	wt	HIS3	hch1::HIS3	MIT
ipG313SΔhch1	G313S	HIS3	hch1::HIS3	MIT
iE381KΔhch1	E381K	HIS3	hch1::HIS3	MIT
ipA587TΔhch1	A587T	HIS3	hch1::HIS3	MIT
i(T/H)* Hsp82p Δhch1	wt	TRP1/HIS3	hch1::TRP1 hch1::HIS3	Shuffling
i(T/H)* Hsp82p^{G313S} Δhch1	G313S	TRP1/HIS3	hch1::TRP1 hch1::HIS3	Shuffling
i(T/H)* Hsp82p^{E381K} Δhch1	E381K	TRP1/HIS3	hch1::TRP1 hch1::HIS3	Shuffling
i(T/H)* Hsp82p^{A587T} Δhch1	A587T	TRP1/HIS3	hch1::TRP1 hch1::HIS3	Shuffling
i(T/H)* His Hsp82p Δhch1	6xHisHsp82	TRP1/HIS3	hch1::TRP1 hch1::HIS3	Shuffling
i(T/H)* His Hsp82p^{G313S} Δhch1	6xHisG313S	TRP1/HIS3	hch1::TRP1 hch1::HIS3	Shuffling
i(T/H)* His Hsp82p^{E381K} Δhch1	6xHisE381K	TRP1/HIS3	hch1::TRP1 hch1::HIS3	Shuffling
i(T/H)* His Hsp82p^{A587T} Δhch1	6xHisA587T	TRP1/HIS3	hch1::TRP1 hch1::HIS3	Shuffling
ip82Δaha1	wt	HIS3	aha1::HIS3	MIT
ipG313SΔaha1	G313S	HIS3	aha1::HIS3	MIT
iE381KΔaha1	E381K	HIS3	aha1::HIS3	MIT
ipA587TΔaha1	A587T	HIS3	aha1::HIS3	MIT

i(T/H)* Hsp82p Δaha1	wt	TRP1/HIS3	aha1::TRP1 aha1::HIS3	Shuffling
i(T/H)* Hsp82p^{G313S} Δaha1	G313S	TRP1/HIS3	aha1::TRP1 aha1::HIS3	Shuffling
i(T/H)* Hsp82p^{E381K} Δaha1	E381K	TRP1/HIS3	aha1::TRP1 aha1::HIS3	Shuffling
i(T/H)* Hsp82p^{A587T} Δaha1	A587T	TRP1/HIS3	aha1::TRP1 aha1::HIS3	Shuffling
i(T/H)* His Hsp82p Δaha1	6xHisHsp82	TRP1/HIS3	aha1::TRP1 aha1::HIS3	Shuffling
i(T/H)* His Hsp82p^{G313S} Δaha1	6xHisG313S	TRP1/HIS3	aha1::TRP1 aha1::HIS3	Shuffling
i(T/H)* His Hsp82p^{E381K} Δaha1	6xHisE381K	TRP1/HIS3	aha1::TRP1 aha1::HIS3	Shuffling
i(T/H)* His Hsp82p^{A587T} Δaha1	6xHisA587T	TRP1/HIS3	aha1::TRP1 aha1::HIS3	Shuffling
iT His Hsp82p pRS426854 vc	6xHisHsp82	TRP1		Shuffling
iT His Hsp82p pRS426854 HCH1myc	6xHisHsp82	TRP1		Shuffling
iT His Hsp82p pRS426854 AHA1myc	6xHisHsp82	TRP1		Shuffling
iT His Hsp82p pRS426854 NtermAHA1myc	6xHisHsp82	TRP1		Shuffling
iT His Hsp82p^{E381K} pRS426854vc	6xHisE381K	TRP1		Shuffling
iT His Hsp82p^{E381K} pRS426854 HCH1myc	6xHisE381K	TRP1		Shuffling
iT His Hsp82p^{E381K} pRS426854 AHA1myc	6xHisE381K	TRP1		Shuffling

iT His Hsp82p^{E381K} pRS426854 NtermAHA1myc	6xHisE381K	TRP1		Shuffling
iT His Hsp82p rdm p41KANTEF vc	6xHisHsp82	TRP1		Shuffling
iT His Hsp82p rdm p41KANTEF HCH1myc	6xHisHsp82	TRP1		Shuffling
iT His Hsp82p Δhch1 p41KANTEF vc	6xHisHsp82	TRP1	hch1::TRP1	Shuffling
iT His Hsp82p Δhch1 p41KANTEF HCH1myc	6xHisHsp82	TRP1	hch1::TRP1	Shuffling
iT His Hsp82p^{G313S} rdm p41KANTEF vc	6xHisG313S	TRP1		Shuffling
iT His Hsp82p^{G313S} rdm p41KANTEF HCH1myc	6xHisG313S	TRP1		Shuffling
iT His Hsp82p^{G313S} Δhch1 p41KANTEF vc	6xHisG313S	TRP1	hch1::TRP1	Shuffling
iT His Hsp82p^{G313S} Δhch1 p41KANTEF HCH1myc	6xHisG313S	TRP1	hch1::TRP1	Shuffling
iT His Hsp82p^{A587T} rdm p41KANTEF vc	6xHisA587T	TRP1		Shuffling
iT His Hsp82p^{A587T} rdm p41KANTEF HCH1myc	6xHisA587T	TRP1		Shuffling
iT His Hsp82p^{A587T} Δhch1 p41KANTEF vc	6xHisA587T	TRP1	hch1::TRP1	Shuffling
iT His Hsp82p^{A587T} Δhch1 p41KANTEF HCH1myc	6xHisA587T	TRP1	hch1::TRP1	Shuffling

*These strains were constructed in two ways – T denotes integration at TRP1, H denotes integration at HIS3

**MIT denotes strains obtained from Susan Lindquist (MIT)

*** Shuffling denotes strains generated by plasmid shuffling into ΔPCLDa donor strain

2.1.5 Antibodies

Our primary antibodies were used in western blotting experiments. Secondary antibodies were obtained from Jackson Labs and were used at a 1:4000 dilution.

Table 2.7: Antibodies used in western blotting

Primary Antibody	Dilution	Type	Secondary	Supplier
Anti-Actin	1:2000	Polyclonal	Goat α Rabbit	Dr. Gary Eitzen
Anti HIS tag	1:1000	Monoclonal	Goat α Mouse	EMD Millipore Corporation
Anti-MYC clone 4A6	1:1000	Monoclonal	Goat α Rabbit	New England BioLabs
C-Myc Antibody (9E10)	1:1000	Monoclonal	Goat α Mouse	Prepared from the 9E10 hybridoma

2.2 Methods

2.2.1 Plasmid Isolation

Our plasmids contain a GPD promoter and CYC terminator cloned out of p414GPD (Mumberg et al. 1995) and into pRS404 or pRS403, generating our p404GPD and p403GPD series. PCR was used to amplify *HCH1* with BglII and XhoI sites, including nested NdeI and BamHI sites respectively, both with and without an N-terminal His-tag. *HCH1* products were then cut with BamHI and XhoI and cloned into either p404GPD or p403GPD generating p404GPDNTHCH1/p403GPDNTHCH1 and p404GPDHisHCH1/p403GPDHisHCH1 respectively. *HSP82,* and mutant $HSP82^{A587T}$, and $HSP82^{G313S}$ gene fragments were cloned NdeI-BamHI into the above vectors in place of *HCH1* to generate the plasmids p404GPDHisHsp82, p404GPDNTHsp82, p404GPDHisA587T, p404GPDNTA587T, p404GPDHisG313S, p404GPDNTG313S,

p403GPDHisHsp82, p403GPDNTHsp82, p403GPDHisA587T, p403GPDNTA587T, p403GPDHisG313S, and p403GPDNTG313S.

Quickchange site-directed mutagenesis was used to introduce another Hsp90 mutant, Hsp82p^{V391E}, into the pET11dHis template plasmids pET11dHisHsp82, pET11dHisA587T, and pET11dHisG313S. Quickchange PCR samples were set up including 5µl 10x reaction buffer, 50ng template pET11dHis DNA, 10pmol primer 0289, 10pmol primer 0290, and 1µl dNTP to a total of 50µl using dd$H_2$0. 1µl PfuTurbo DNA polymerase was added and samples were run in PCR. Following PCR, 1µl of DPN1 enzyme was added to each sample and incubated at 37°C for 4hours. *HSP82^{V391E}, HSP82$^{V391E-A587T}$,* and *HSP82$^{V391E-G313S}$* gene fragments were cloned NdeI-BamHI into the p404GPDNTHCH1 and p404GPDHisHCH1 vectors in place of *HCH1* to generate the plasmids p404GPDHisHsp82^{V391E}, p404GPDNTHsp82 ^{V391E}, p404GPDHisHsp82$^{V391E-A587T}$, p404GPDNTHsp82 $^{V391E-A587T}$, p404GPDHisHsp82$^{V391E-G313S}$, and p404GPDNTHsp82 $^{V391E-G313S}$.

The galactose inducible Over-expression pRS426854, pRS424854 and pRS423854 plasmids were generated by cloning the GAL1/10 promoter into pRS426, pRS424 and pRS423 respectively, using EcoRI-BamHI. PCR was used to amplify *HCH1* and *AHA1* gene fragments containing both a C-terminal myc tag or no tag then cloned into pRS426854, pRS424854, or pRS423854 downstream of the GAL1 promoter using BamHI-SacI for *HCH1* and *AHA1$^{1-156}$* or BamHI-NotI for *AHA1*.

The Geneticin(G418)-selectable Over-expression plasmids were generated by cloning the TEF and GPD promoters into pRS41KAN and pRS42KAN (Mumberg et al. 1995; Taxis et al. 2006) using KpnI-SacI in restriction endonuclease digests to generate pRS41KANTEF, pRS41KANGPD, pRS42KANTEF, and pRS42KANGPD. PCR was used to

amplify *HCH1myc* and *AHA1myc* gene fragments which were then digested along with p41KANTEF, p41KANGPD, p42KANTEF, and p42KANGPD using BamHI-XhoI. The *HCH1myc* and *AHA1myc* gene fragments were cloned into the G418-selectable series downstream of the G418 driven TEF or GPD promoter by ligation, generating p41KANTEFHCH1myc, p41KANTEFAHA1myc, p41KANGPDHCH1myc, p41KANGPDAHA1myc, p42KANTEFHCH1myc, p42KANTEFAHA1myc, p42KANGPDHCH1myc, p42KANGPDAHA1myc.

2.2.2 Restriction Endonuclease Digestion

We digested DNA with the restriction endonucleases indicated. 5µg of DNA was digested in a final volume of 50µl (5µl NEB buffer, 5µl 10XBSA, 2µl Endonuclease, 5µg DNA, and remaining µl dH_2O). The p404 plasmids were linearized using HindIII to digest prior to being introduced into yeast cells by plasmid shuffling. The p403 plasmids were linearized using NheI to digest prior to being introduced into yeast cells by plasmid shuffling. Restriction endonuclease reactions were set up according to New England Biolabs protocols.

Table 2.8: Restriction Endonucleases

Restriction Endonuclease	Other Components	Use
BamHI-EcoRI	NEB#3 + BSA	Plasmid isolation
BamHI-KpnI	NEB#1 + BSA	Plasmid isolation
BamHI-NdeI	NEB#4 + BSA	Plasmid isolation
BamHI-NotI	NEB#3 + BSA	Plasmid isolation
BamHI-NotI HF	NEB#4 + BSA	Plasmid isolation
BamHI-XhoI	NEB#3 + BSA	Plasmid isolation

DPN1	NEB#4	Quickchange mutagenesis
HindIII	NEB#2	Linearize p404GPD plasmids
KpnI-SacI	NEB#1 + BSA	Plasmid isolation
NheI	NEB#2 + BSA	Linearize p403GPD plasmids

2.2.3 Polymerase Chain Reaction

Polymerase chain reaction was utilized to amplify gene segments in accordance with the TopTaq PCR handbook.

2.2.4 Agarose Gel Electrophoresis

PCR DNA products and restriction endonuclease digest DNA products were separated by agarose gel electrophoresis. Agarose gels were run at 0.8% (1.6g Agarose and 200mL 1X TAE) and samples were run against a 1Kb DNA ladder. Gels were visualized using the FluorChemQ system and DNA samples were then cut from the gel and agarose gel purified. Following PCR if the entire DNA sample was run through an agarose gel then the DNA was cut out and purified by agarose gel purification. If only a small amount of the PCR sample was run on agarose gel to check the size of the DNA within the sample then the remainder of the sample was purified by PCR purification.

2.2.5 Purification of DNA Fragments

PCR purification of DNA fragments following PCR follows the PCR Purification Kit Protocol (QIAGEN). Five volumes of Buffer PB was added to one volume of PCR sample which was then placed in a QIAquick spin column and spun at 4°C using an eppendorf

centrifuge 5417R with an F45-30-11 rotor set at 14500rpm for 10 minutes. Flowthrough was discarded then 750µl Buffer PE with ethanol was added and pelleted using an eppendorf centrifuge 5417C with an F45-30-11 rotor set at 13500 rpm for 60 seconds. Flowthrough was discarded again and the QIAquick spin column was moved to a clean 1.5mL microcentrifuge tube. DNA was eluted with 50µl of dH_2O and spun using an eppendorf centrifuge 5417C with an F45-30-11 rotor set at 13500rpm for 60 seconds.

Agarose gel purification of DNA fragments following Agarose Gel Electrophoresis follows and the QIAquick gel extraction kit Protocol. Gel slices were cut from the agarose gel and placed in microcentrifuge tubes. Five volumes of Buffer QG was added to one volume of agarose gel sample then tubes were placed in the $50^{\circ C}$ heat rack for 10 minutes or until the gel was melted fully. The sample mixture was then added to QIAquick spin column and spun using an eppendorf centrifuge 5417C with an F45-30-11 rotor set at 13500rpm for 60 seconds. Flowthrough was discarded then 500µl Buffer QG was added and spun using an eppendorf centrifuge 5417C with an F45-30-11 rotor set at 13500rpm for 60 seconds. Flowthrough was discarded then 750µl Buffer PE with ethanol was added and spun using an eppendorf centrifuge 5417C with an F45-30-11 rotor set at 13500rpm for 60 seconds. Flowthrough was discarded and samples were spun using an eppendorf centrifuge 5417C with an F45-30-11 rotor set at 13500rpm for 60 seconds. Flowthrough was discarded again and the QIAquick spin column was moved to a clean 1.5mL microcentrifuge tube. DNA was eluted with 30µl of dH_2O and left to incubate at room temperature for 5 minutes then spun using an eppendorf centrifuge 5417C with an F45-30-11 rotor set at 13500rpm for 60 seconds.

2.2.6 Ligation of DNA

Ligation mixtures included a 1:3 ratio of vector DNA to insert DNA up to 17µl. Control samples contained a 1:3 ratio of vector DNA to dH_2O up to 17µl in the same amounts as the vector-insert samples. 2µl T4 DNA Ligase Buffer and 1µl T4 DNA Ligase was added to these samples to a total of 20µl. Ligation samples were incubated for a minimum of one hour at room temperature.

2.2.7 Transformation of *Escherichia coli*

Following a one hour ligation of gene fragments 2µl of plasmid DNA was added to 100µl DH5α competent *Escherichia coli* cells. The mix was left on ice for 20mins followed by 45 second heat shock at $40°^C$ and 2 minutes on ice again. Samples were then removed from ice and 1mL of LB medium was added before a 30 minute recovery at 37°C. Samples were pulse spun in the microcentrifuge, resuspended, and grown on LB-AMP plates overnight at 37°C.

2.2.8 Transformation of *Saccharomyces cerevisiae*

Lithium acetate transformation was utilized for transformation of *Saccharomyces* cerevisiae. Lithium Acetate (LiAcTE) (100mM LiAc, 10mM Tris pH7.2, and 1mM EDTA) was implemented in disruption of the yeast cell wall. *S. cerevisiae* cultures were grown in specified liquid medium overnight spinning at 30°C. Optical density (O.D.) readings of the cultures were taken to determine the absorbance readings of cell culture samples. Cultures were diluted to 0.3 O.D. and grown spinning at 30°C for 4 hours. The first step of the three-step transformations consisted of taking a 1mL sample of each culture, spin using an eppendorf centrifuge 5417C with an F45-30-11 rotor set at

13500rpm for 60 seconds, re-suspended in 1mL of dH_2O, spin using an eppendorf centrifuge 5417C with an F45-30-11 rotor set at 13500rpm for 60 seconds, re-suspended in 1mL of LiAcTE, and spin using an eppendorf centrifuge 5417C with an F45-30-11 rotor set at 13500rpm for 60 seconds. Step two consisted of transformation with salmon sperm DNA, 1-2ug DNA, PEG (LiACTE and 40%PEG3550) and DMSO. The recovery step consisted of a one hour incubation in a 30°C water bath, 15 minutes at 42°C, re-suspension in 1M sorbitol, and addition of 1mL of YPD medium. YPD-sorbitol liquid culture transformations were incubated overnight spinning at 30°C then plated on specified medium plates in order to ensure the *S. cerevisiae* strains had taken up the specific gene fragment or plasmid during transformation.

2.3 Construction of Yeast Strains

2.3.1 ΔPCLDa Knock Out Strains

The pRS403 and pRS404 template plasmids were used to amplify the HIS3 and TRP1 gene fragments respectively using flanking sequences from *AHA1* and *HCH1* via PCR (TopTaq PCR Handbook). PCR-mediated gene disruption and homologous recombination were implemented in the replacement of *HCH1* or *AHA1* genes in the chromosome of the ΔPCLDa yeast strain with *HIS3* or *TRP1* to create the ΔPCLDa (HIS)AHA1, ΔPCLDa ΔAHA1::HIS3, and ΔPCLDa ΔHCH1::HIS3 or the ΔPCLDa (TRP)AHA1, ΔPCLDa ΔAHA1::TRP1, and ΔPCLDa ΔHCH1::TRP1 strains respectively.

2.3.2 MIT Knock Out Yeast Strains

We obtained the MIT strains used in previous experiments performed by Nathan *et al.* (Nathan et al. 1995) from Dr Susan Lindquist's lab.

TRP1 and *HIS3* gene fragments were amplified off of the pRS404 and pRS403 template plasmids respectively using flanking sequences from *AHA1* and *HCH1* in PCR (TopTaq PCR Handbook). PCR-mediated gene disruption and homologous recombination was implemented to construct the knock out chromosome strains by replacing *HCH1* and *AHA1* genes with the *TRP1* or *HIS3* gene fragments generating p404Δhch1 and p404Δaha1, or p403Δhch1 and p403Δaha1 plasmids respectively. Single knock out strains were created through Lithium Acetate Transformation using the p403 plasmids and were selected for by histidine autotrophy on SC-HIS plates for the ip82Δhch1, ip82Δaha1, ipA587TΔhch1, ipA587TΔaha1, ipG313SΔhch1 and ipG313SΔaha1 strains.

The double knock-out strains were created through Lithium Acetate Transformation of the ip82Δaha1, ipA587TΔaha1, and ipG313SΔaha1 strains grown in SC-HIS medium. Following transformation, cultures were plated on SC-HIS-TRP in order to ensure the *S. cerevisiae* single knock out chromosomes had taken up the *TRP1* gene fragment in place of the *HCH1* gene fragment. Colony diagnostic PCR was utilized to confirm the double knock out transformants.

2.3.3 iT and iH Knock Out Yeast Strains

For the iT strains, HindIII digests were used to linearize the p404GPDHisHsp82, p404GPDHsp82, p404GPDHisA587T, p404GPDA587T, p404GPDHisG313S, and p404GPDG313S vectors which were then shuffled into the ΔPCLDa (HIS)AHA1, ΔPCLDa ΔAHA1::HIS3, and ΔPCLDa ΔHCH1::HIS3 strains described in. The linearized p404GPD plasmids with a functional TRP locus were shuffled into the non-functional TRP locus of the chromosome of the ΔPCLDa strains using Lithium Acetate Transformation creating the yeast iT strains known as iTNTWTa, iTNTWTΔAHA1a, iTNTWTΔHCH1a, iTNTA587Ta,

iTNTA587TΔAHA1a, iTNTA587TΔHCH1a, iTNTG313Sa, iTNTG313SΔAHA1a, iTNTG313SΔHCH1a, iTHisWTa, iTHisWTΔAHA1a, iTHisWTΔHCH1a, iTHisA587Ta, iTHisA587TΔAHA1a, iTHisA587TΔHCH1a, iTHisG313Sa, iTHisG313SΔAHA1a, and iTHisG313SΔHCH1a. The transformation cultures were then grown on SC-TRP+URA+1g 5FOA/L medium plates to select for successful plasmid shuffling.

The pRS406 template plasmid was used to amplify the URA3 gene fragment using the flanking sequences from *AHA1* and *HCH1* via PCR (TopTaq PCR Handbook). The double knock-outs were created using Lithium Acetate Transformation with the yeast iTΔaha1 strains grown in liquid SC-TRP. Following transformation these double knock-out cultures were grown on SC-TRP-URA plates to ensure the URA3 gene fragment had been taken into the chromosome of the *S. cerevisiae* single knock out strains in place of the *HCH1* gene by both tryptophan and uracil autotrophy. Single and double knock outs were confirmed by colony diagnostic PCR and agarose gels.

The iH yeast strains were constructed by Lithium Acetate Transforming the HindIII linearized p403GPDHisHsp82, p403GPDHsp82, p403GPDHisA587T, p403GPDA587T, p403GPDHisG313S, and p403GPDG313S vectors with a functional His locus into the ΔPCLDa(TRP)AHA1, ΔPCLDa ΔAHA1::TRP1, and ΔPCLDa ΔHCH1::TRP1 strains containing a non-functional His locus. This LiAcT yielded the iH yeast strains iHNTWTa, iHNTWTΔAHA1a, iHNTWTΔHCH1a, iHNTA587Ta, iHNTA587TΔAHA1a, iHNTA587TΔHCH1a, iHNTG313Sa, iHNTG313SΔAHA1a, iHNTG313SΔHCH1a, iHHisWTa, iHHisWTΔAHA1a, iHHisWTΔHCH1a, iHHisA587Ta, iHHisA587TΔAHA1a, iHHisA587TΔHCH1a, iHHisG313Sa, iHHisG313SΔAHA1a, and iHHisG313SΔHCH1a. The transformation cultures were then grown on SC-HIS+URA+1g 5FOA/L medium plates to select for successful plasmid shuffling.

The pRS404 template plasmid was used to amplify the TRP1 gene fragment using the flanking sequences from *AHA1* and *HCH1* via PCR (TopTaq PCR Handbook). The double knock-outs were created using Lithium Acetate Transformation with the yeast iHΔaha1 strains grown in liquid SC-HIS. Following transformation these double knock-out cultures were grown on SC-HIS-TRP plates to ensure the TRP1 gene fragment had been taken into the chromosome of the *S. cerevisiae* single knock out strains in place of the *HCH1* gene by both histidine and tryptophan autotrophy. Single and double knock outs were confirmed by colony diagnostic PCR and agarose gels.

2.3.4 iT and iH GAL-Inducible Over-expression Strains

The over-expression system used in the iTNTWTa, iTNTE381Ka, iTHisWTa, and iTHisE381Ka strains is driven by the GAL-inducible expression system. Lithium Acetate Transformation was used to transform the pRS426854 Hch1p, Aha1p and Hch1p/Aha1p over-expression plasmids into the associated iT strains. To ensure the pRS426854 GAL-inducible plasmid had been taken into the iT *S. cerevisiae* cells, the cultures were grown on SC-TRP-URA plates following transformation.

2.3.5 iT Drug-Selectable Over-expression Strains

The over-expression system used in the iTHisWTa, iTHisA587Ta, and iTHisG313Sa strains is driven by the Geneticin(G418)-selectable expression system. Lithium Acetate Transformation was used to transform the p41KANTEF, p41KANGPD, p42KANTEF, p42KANGPD, p41KANTEFHCH1myc, p41KANTEFAHA1myc, p41KANGPDHCH1myc, p41KANGPDAHA1myc, p42KANTEFHCH1myc, p42KANTEFAHA1myc, p42KANGPDHCH1myc, and p42KANGPDAHA1myc over-

expression plasmids into the associated iT strains. To ensure the G418-selectable plasmids had been taken into the iT *S. cerevisiae* cells, the cultures were grown on YPD+200mg G418/L medium plates following transformation.

2.3.6 iT Hsp82p^{V391E} Strains

For the iT strains, HindIII digests were used to linearize the p404GPDHisHsp82^{V391E}, p404GPDNTHsp82^{V391E}, p404GPDHisHsp82p$^{V391E\text{-}A587T}$, p404GPDNTHsp82p$^{V391E\text{-}A587T}$, p404GPDHisHsp82p$^{V391E\text{-}G313S}$, and p404GPDNTHsp82p$^{V391E\text{-}G313S}$ vectors which were then shuffled into the ΔPCLDa strain. The linearized p404GPD plasmids with a functional TRP locus were shuffled into the non-functional TRP locus of the chromosome of the ΔPCLDa strain using Lithium Acetate Transformation creating the yeast iT strains known as iTHisHsp82^{V391E}, iTNTHsp82^{V391E}, iTHisHsp82$^{V391E\text{-}A587T}$, iTNTHsp82$^{V391E\text{-}A587T}$, iTHisHsp82$^{V391E\text{-}G313S}$, and iTNTHsp82$^{V391E\text{-}G313S}$. The transformation cultures were then grown on SC-TRP plates to select for successful plasmid shuffling.

2.4 Yeast Growth Assays

2.4.1 Growth Plating Assays

Knock-out yeast strains from the MIT and iT series were grown in liquid YPD medium and incubated spinning overnight at 30°C. These cultures were then measured using 600.0nm O.D. readings and were ten-fold serial diluted beginning with an initial dilution of 0.1 O.D. units. Culture dilutions were plated in 5µl spots on YPD medium agar plates or YPD+17AAG plates (150µM, 300µM, and 450µM 17AAG) or YPD+NVP plates (50µM, 200µM, and 350µM NVP). YPD dilution plates were grown overnight at room

temperature, 30°C, 34.5°C, and 37°C, while YPD+17AAG plates and YPD+NVP plates were grown overnight at 30°C.

GAL-inducible over-expression strains from the iT series were grown in liquid SC-TRP-URA medium and incubated spinning overnight at 30°C. These cultures were then measured using 600.0nm O.D. readings and were ten-fold serial diluted beginning with an initial dilution of 0.1 O.D. units. iT culture dilutions were plated in 5µl spots on galactose vs glucose SC-TRP-URA medium plates. Dilution plates were grown overnight at 30°C, 34.5°C, 35.5°C and 37°C.

G418-selectable over-expression strains from the iT series were grown in liquid YPD+200mg G418/L medium and incubated spinning overnight at 30°C. These cultures were then measured using 600.0nm O.D. readings and were ten-fold serial diluted beginning with an initial dilution of 0.1 O.D. units. iT culture dilutions were plated in 5µl spots on YPD+200mg G418/L medium plates and YPD+G418+NVP medium plates (50µM, 200µM, and 350µM NVP). YPD dilution plates were grown overnight at 30°C, 34.5°C, 35.5°C and 37°C, while YPD+G418+NVP plates were grown overnight at 30°C.

2.4.2 Growth Liquid Assays

Knock-out yeast strains from the iT series were grown in liquid YPD medium and incubated spinning overnight at 30°C. These cultures were then measured using 600.0nm O.D. readings and were diluted to an initial dilution of 0.01 O.D. units and grown for 24 hours in liquid YPD medium at both 30°C and 35.5°C taking 600.0nm O.D. readings at 4 hours, 12 hours, and 24 hours. These readings were then charted using the Prism computer graphing program.

2.5 Western Blotting

2.5.1 Yeast Protein Preparation by Alkaline Lysis

The iT knock out strain cells were grown in YPD medium overnight. The following day, the cultures were measured using 600.0nm O.D. readings then were diluted to 0.2 O.D. units and grown for both 7 hours and overnight at both 30°C and 37°C. 0.5 O.D. units of each sample were transferred tomicrocentrifuge tubes and spun using an eppendorf centrifuge 5417C with an F45-30-11 rotor set at 3000rpm for 2 minutes. Pellets were resuspended in dH_2O and spun using an eppendorf centrifuge 5417C with an F45-30-11 rotor set at 3000rpm for two minutes. Pellets were resuspended in 500µl dH_2O and 90µl of lysis buffer (Table 2.3) was added. Samples were vortexed for 30 seconds each a total of two times then incubated on ice for 10 minutes. Following incubation on ice 250µl of 100% TCA was added to samples which were then vortexed for 10 seconds each then incubated on ice for 10 minutes. Following incubation on ice the samples were spun at 4°C using an eppendorf centrifuge 5417R with an F45-30-11 rotor set at 14500rpm for 10 minutes then supernatant was removed by vacuum and pellets were washed in 500µl of 80% acetone on ice for 10 minutes. Following incubation on ice the samples were spun at 4°C using an eppendorf centrifuge 5417R with an F45-30-11 rotor set at 14500rpm for 10 minutes then supernatant was removed by vacuum and pellets were left to dry at room temperature for 30 minutes. Samples were then resuspended in 50µl 2x sample buffer (Table 2.3). Samples were then run on SDS PAGE.

The iT GAL-inducible over-expression strains were grown in SC-TRP-URA+RAF overnight. The following day, the cells were diluted to an O.D of 0.2 and incubated over night with in SC-TRP-URA+GLU and SC-TRP-URA+GAL at both 30°C and 37°C. 0.5 O.D.

units of each sample was transferred to microcentrifuge tubes and spun using an eppendorf centrifuge 5417C with an F45-30-11 rotor set at 3000rpm for 2 minutes. Pellets were resuspended in dH_2O and spun using an eppendorf centrifuge 5417C with an F45-30-11 rotor set at 3000rpm for 2 minutes. Pellets were resuspended in 500µl dH_2O and 90µl of lysis buffer (Table 2.3) was added. Samples were vortexed for 30 seconds each a total of two times then incubated on ice for 10 minutes. Following incubation on ice 250µl of 100% TCA was added to samples which were then vortexed for 10 seconds each then incubated in ice for 10 minutes. Following incubation on ice the samples were spun at 4°C using an eppendorf centrifuge 5417R with an F45-30-11 rotor set at 14500rpm for 10 minutes then supernatant was removed by vacuum and pellets were washed in 500µl of 80% acetone on ice for 10 minutes. Following incubation on ice the samples were spun at 4°C using an eppendorf centrifuge 5417R with an F45-30-11 rotor set at 14500rpm for 10 minutes then supernatant was removed by vacuum and pellets were left to dry at room temperature for 30 minutes. Samples were then resuspended in 50µl 2x sample buffer (Table 2.3). Samples were then run on SDS PAGE.

The iT G418 over-expression strains were grown in YPD+200mg G418/L medium overnight. 0.5 O.D. units of each sample was transferred to microcentrifuge tubes and spun using an eppendorf centrifuge 5417C with an F45-30-11 rotor set at 3000rpm for 2 minutes. Pellets were resuspended in dH_2O and spun using an eppendorf centrifuge 5417C with an F45-30-11 rotor set at 3000rpm for two minutes. Pellets were resuspended in 500µl dH_2O and 90µl of lysis buffer (Table 2.3) was added. Samples were vortexed for 30 seconds each a total of two times then incubated on ice for 10 minutes. Following incubation on ice 250µl of 100% TCA was added to samples which were then

vortexed for 10 seconds each then incubated in ice for 10 minutes. Following incubation on ice the samples were spun at 4°C using an eppendorf centrifuge 5417R with an F45-30-11 rotor set at 14500rpm for 10 minutes then supernatant was removed by vacuum and pellets were washed in 500µl of 80% acetone on ice for 10 minutes. Following incubation on ice the samples were spun at 4°C using an eppendorf centrifuge 5417R with an F45-30-11 rotor set at 14500rpm for 10 minutes then supernatant was removed by vacuum and pellets were left to dry at room temperature for 30 minutes. Samples were then resuspended in 50µl 2x sample buffer (Table 2.3). Samples were then run on SDS PAGE.

2.5.2 Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Following sodium hydroxide sample preparation cell pellets were resuspended in 500µl SDS-PAGE sample buffer (Table 2.3). Samples were heated at 100°C for 10 minutes then vortexed for 10 seconds and spun at 4°C using an eppendorf centrifuge 5417R with an F45-30-11 rotor set at 14500rpm for 10 minutes. 15µl of sample was separated on 10% SDS-PAGE running gel for knock out series and 12% SDS-PAGE running gel for over-expression series.

2.5.3 Western Transfer and Immunoblot

Following SDS-PAGE, protein samples were transferred to pure nitrocellulose membrane (BIO RAD). Membranes were blocked overnight for non-specific binding in 3% BSA (Tris-buffered saline (TBS), 0.1% Tween, and 3% Bovine Serum Albumin). His-tagged Hsp82 and myc-tagged co-chaperone levels were detected by immunoblotting.

Hsp82 was probed for using the Anti-His Tag Rabbit (1:1000 dilution) antibody (Cell Signaling) overnight at 4°C followed by a HRP-conjugated anti-rabbit IgG (1:4000 dilution). The co-chaperones were probed for using the Anti-myc Tag clone 4A6 (1:1000 dilution) antibody overnight at 4°C followed by a HRP-conjugated anti-mouse IgG (1:4000 dilution). Protein expression levels were controlled for by probing with Anti-Actin (1:2000 dilution) antibody overnight at 4°C followed by a HRP-conjugated anti-rabbit IgG (1:4000 dilution). Specific bands were detected by electrochemiluminescence (ECL). Western membranes were imaged using the FluorChemQ system.

CHAPTER THREE

Results – The Co-chaperone Hch1p Regulates Sensitivity to the Hsp90 Inhibitor NVP-AUY922 in Yeast

A part of this chapter, as indicated in the text, has been published in

Armstrong, H., A. Wolmarans, et al. (2012). "The Co-Chaperone Hch1 Regulates Hsp90 Function Differently than Its Homologue Aha1 and Confers Sensitivity to Yeast to the Hsp90 Inhibitor NVP-AUY922." PLoS One **7**(11): e49322.

3.1 Initial Screening Revealed Growth of Yeast Strains Expressing Hsp90 Mutants, Hsp82p^{A587T} or Hsp82p^{G313S}, is Restored by Deletion of Hsp90 Co-chaperone Hch1p.

We began our work with eight temperature-sensitive yeast strains provided to us by Dr. Susan Lindquist (Nathan et al. 1995), that each expressed either the wild-type Hsp82p, or a different mutant form of Hsp82p as their sole source of Hsp90. The mutants included Hsp82p^{T22I}, Hsp82p^{A41V}, Hsp82p^{G81S}, Hsp82p^{T101I}, Hsp82p^{G170D}, Hsp82p^{G313S}, Hsp82p^{E381K}, and Hsp82p^{A587T} (Figure 1.5; (Nathan et al. 1995)). Previous studies have shown that over-expression of the Hsp90 co-chaperone *HCH1* is capable of restoring growth in specific Hsp90 mutant strains (Nathan et al. 1999). Over-expression of the multicopy suppressor *HCH1* impairs the growth of yeast strains expressing the mutants Hsp82p^{G313S} or Hsp82p^{A587T} (Nathan et al. 1999).

3.2 *HCH1* Knock Out Restores Growth of Yeast Expressing the Hsp90 Mutants Hsp82p^{A587T} or Hsp82p^{G313S}.

We hypothesized that knock out of *HCH1* would have the same effect as knock out of *AHA1*, in the eight Hsp90 mutant strains obtained from Dr. Susan Lindquist, as these two co-chaperones are considered to be homologous (Dr. Susan Lindquist, MIT). To test this hypothesis, we deleted either the Hsp90 co-chaperone *HCH1*, or its homologue *AHA1* in the wild-type strain, ip82, and eight *ts* mutant yeast strains and examined their growth properties at elevated temperatures (Table 1.1). Surprisingly, we observed that, of the eight *ts* mutant strains, two strains, ipA587T and ipG313S, had restored growth when *HCH1* was deleted but not when *AHA1* was deleted, therefore, we continued our research with these two mutant strains (Armstrong et al. 2012). This

data suggests that the deletion of *HCH1* was able to restore growth in these mutant strains because Hch1p competitively binds with Aha1p for the middle domain of Hsp90.

We expected that if Hch1p was preventing a function of Aha1p from occurring by competitively binding Hsp90M, then deletion of both *HCH1* and *AHA1* would no longer be able to restore growth in these mutant strains. To test this hypothesis we introduced double knock out *HCH1/AHA1* ipG313S and ipA587T strains. Growth assays on the ip82, ipA587T and ipG313S *S. cerevisiae Δhch1, Δaha1*, and *Δhch1/aha1* strains showed that, at 30°C and 35.5°C, the mutant strains containing *Δhch1, Δaha1*, and *Δhch1/aha1* knock outs grew to the same level as the ip82 strains (Figure 3.1A (Armstrong et al. 2012)). Deletion of *HCH1* rescued growth of the *ts* mutant ipA587T, and slightly rescued ipG313S at 37°C (Figure 3.1A (Armstrong et al. 2012)). The double knock out, *Δhch1/aha1*, was still capable of restoring growth in both the ipA587T and ipG313S *ts* strains at 37°C (Figure 3.1A (Armstrong et al. 2012)). We therefore concluded that restoration of growth of the yeast strains containing the Hsp90 mutants, $Hsp82p^{A587T}$ or $Hsp82p^{G313S}$, by deletion of *HCH1* is not due to a competitive binding between Hch1p and Aha1p for Hsp90M.

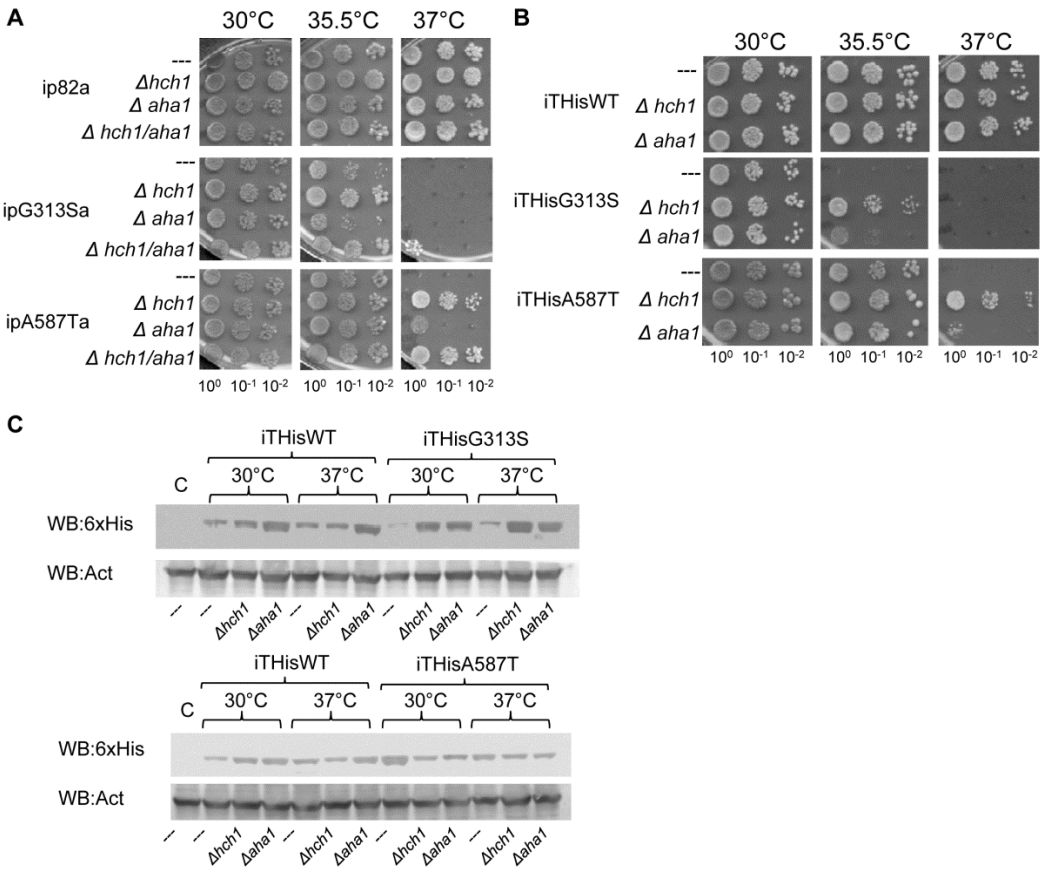

Figure 3.1: Deletion of *HCH1* but not its homologue *AHA1* restores growth at high temperature in the *S. cerevisiae* strains expressing Hsp82p^{A587T} or Hsp82p^{G313S} as their sole source of Hsp90. Cultures were grown in YPD medium overnight at 30°C. Cultures were diluted to 1 x 10^8 cells per mL and 10-fold serial dilutions were prepared. 5µl of each serial dilution were spotted on YPD medium agar plates. (A) Viability of yeast strains, ip82, ipA587T, and ipG313S, obtained from Dr. Susan Lindquist, grown for 2 days at 30°C, 35.5°C, and 37°C. (B) Viability of yeast strains, iTHisHsp82p, iTHisHsp82p^{G313S}, and iTHisHsp82p^{A587T}, that we created, grown for 2 days at 30°C, 35.5°C, and 37°C. (C) Western blot analysis of yeast strains examined in figure 3.1B with anti-His-tag and anti-actin antibodies. Levels of Hsp82p were examined at both 30°C and 37°C, as indicated, in yeast strains expressing NTHsp82p (lane 1 top and bottom), HisHsp82p (lanes 2-7 top and bottom), HisHsp82p^{G313S} (lanes 8-13 top), or HisHsp82p^{A587T} (lanes 8-13 bottom) with *HCH1* deletion (lanes 3, 6, 7, 9, and 12) or *AHA1* deletion (lanes 4, 7, 10, and 13). Figure published by Armstrong *et al.* (Armstrong et al. 2012).

We next wanted to rule out the possibility that deletion of *HCH1* in these mutant strains was capable of restoring growth due to an increase in the levels of Hsp82p in the *Δhch1* strains. In order to perform western blots for the detection of Hsp82p in these cell lines, we decided to construct a new line of yeast strains containing an epitope-tagged Hsp82p, Hsp82p^{A587T}, or Hsp82p^{G313S} as their sole source of Hsp90, as there is currently a lack of a good antibody that recognizes Hsp82p. This series of yeast strains was named the iT series and was constructed by PCR mediated gene disruption and plasmid shuffling as explained in the methods section (Section 2.3.3). Upon repeating the growth assays with our new epitope-tagged iT strains we observed a less striking growth phenotype compared to Dr. Susan Lindquists strains (Figure 3.1B (Armstrong et al. 2012)). Growth assays on the iTHisHsp82^{WT}, iTHisHsp82^{A587T} and iTHisHsp82^{G313S} *S. cerevisiae Δhch1* and *Δaha1* strains showed that at 30°C and 35.5°C, yeast expressing Hsp82p^{A587T} with either *HCH1* or *AHA1* deleted, grew to the same level as the WT strains (Figure 3.1B (Armstrong et al. 2012)). At 30°C the yeast expressing Hsp82p^{G313S} with either *HCH1* or *AHA1* deleted, grew to the same level as the Hsp82p strains (Figure 3.1B (Armstrong et al. 2012)). Deletion of *HCH1* restored growth to the yeast expressing the *ts* mutant Hsp82p^{A587T} up to 37°C, and restored growth to yeast expressing the *ts* mutant Hsp82p^{G313S} up to 35.5°C (Figure 3.1B (Armstrong et al. 2012)). We determined the minor difference between Dr. Susan Lindquist's strains and our iT strains is due to the presence of the epitope-tag on Hsp82p. As our strains containing tagged Hsp82p were still *ts* and their growth was still restored by the same method we continued our experiments with these new strains.

3.3 Hsp82 Expression Level is Not Responsible for Growth Restoration of Yeast Expressing the Hsp90 Mutants, Hsp82p^{A587T} or Hsp82p^{G313S}.

In order to rule out the possibility that deletion of *HCH1* in the iTHisHsp82p^{A587T} and iTHisHsp82^{G313S} strains was capable of restoring growth due to an increase in the levels of Hsp82p, we performed western blots against His-tagged Hsp82p (Figure 3.1C (Armstrong et al. 2012)). Levels of Hsp82p were examined in the iTHisHsp82p, iTHisHsp82p^{A587T}, and iTHisHsp82p^{G313S} co-chaperone knock out strains at both 30°C and the restrictive temperature of 37°C. Actin control revealed that iTHisHsp82p and mutant, iTHisHsp82p^{A587T}, and iTHisHsp82p^{G313S} strains had comparable levels of Hsp82p at both 30°C and 37°C with the exception of the iTHisHsp82p^{G313S} *Δhch1* and *Δaha1* strains (Figure 3.1C (Armstrong et al. 2012)). The iTHisHsp82p^{G313S} *Δhch1* and *Δaha1* strains showed a significant increase in Hsp82p compared to the iTHisHsp82p^{G313S} cells in which co-chaperones were not deleted (Figure 3.1C (Armstrong et al. 2012)). As deletion of *AHA1* had no effect on restoring the growth of these *ts* mutants, the increase in Hsp82p in both knock out strains allows us to conclude that this increase in Hsp82p does not account for the restoration of the growth phenotype in the *Δhch1* strains (Armstrong et al. 2012).

3.4 Deletion of *HCH1* in Yeast Strains Expressing Hsp90 Mutants, Hsp82p^{A587T} or Hsp82p^{G313S}, Increases Growth Rate in Log Phase at High Temperatures.

Growth assays on agar plates are more qualitative than quantitative. Plating allows us to visually assess the growth of our strains by colour and relative size and number of colonies. In contrast, growth assays in liquid allow us to determine the specific properties of the lag, log and stationary phases of growth in our strains, and to

measure the density of cells at different time points. Hsp90 mutants may not grow to the same level as wild-type due to a longer lag time, lower stationary phase, or perhaps they grow slower in log phase. In order to determine the level of growth restoration when *HCH1* was deleted in the iTHisHsp82p^{A587T} and iTHisHsp82p^{G313S} strains compared to iThisHsp82p, we performed growth assays in liquid cultures (Figure 3.2). As was found in the growth assays on agarose plates, at 30°C the *Δhch1* and *Δaha1* iTHisHsp82p^{A587T} and iTHisHsp82p^{G313S} strains grew to the same level as the iTHisHsp82p strains (Figure 3.2). At 35.5°C *Δhch1* restored iTHisHsp82p^{A587T} yeast strain growth levels up to iTHisHsp82p growth levels while iTHisHsp82p^{G313S} cell growth was restored up to half of the growth level of iTHisHsp82p strains (Figure 3.2). Growth assays in liquid also showed that cells expressing Hsp82p^{A587T} or Hsp82p^{G313S} grow slower than iTHisHsp82p strains at high temperatures (Figure 3.2). More time points would need to be examined between 4 hours and 12 hours in order to be able to truly conclude that our mutant strains do not have a longer lag time than wild-type yeast, and that they do not have a lower stationary phase. We currently believe that cells expressing Hsp82p^{A587T} or Hsp82p^{G313S} grow slower in log phase but do not have a longer lag time or lower stationary phase than cells expressing Hsp82p.

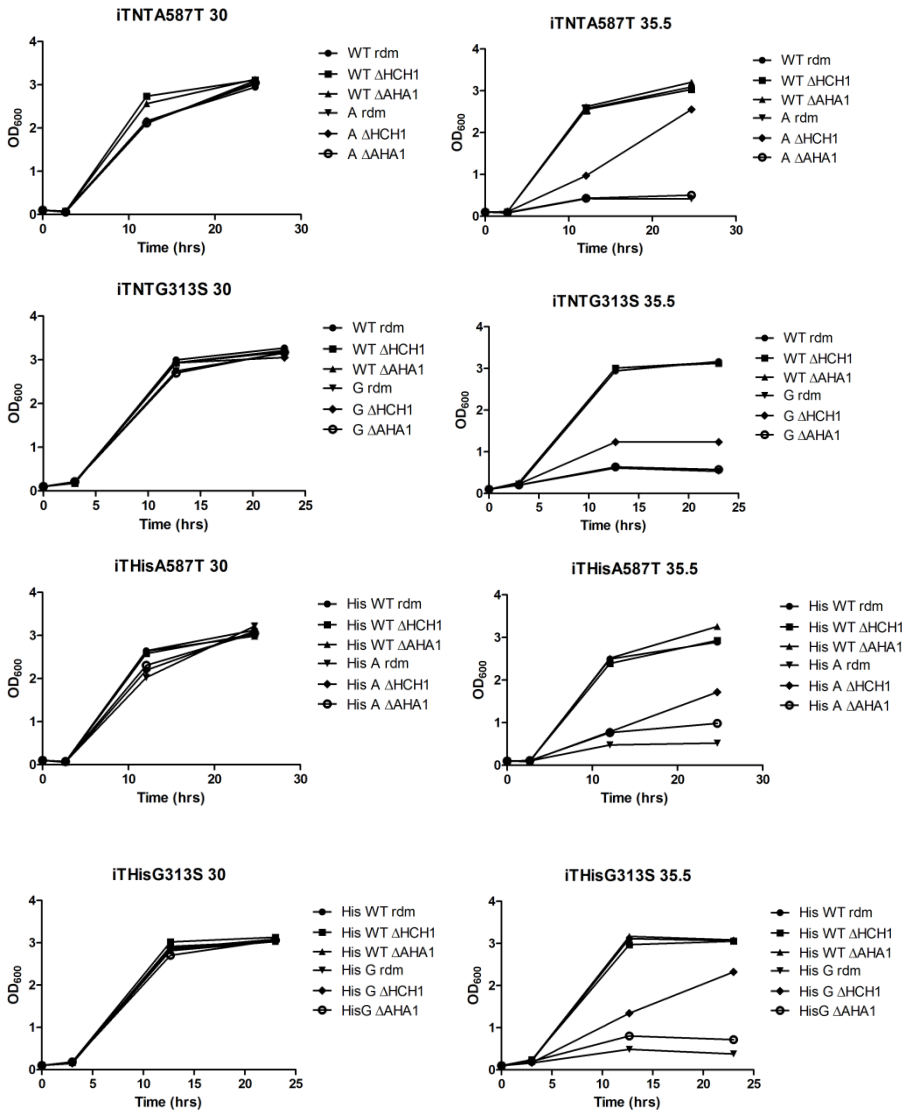

Figure 3.2: Deletion of *HCH1*, but not *AHA1*, restores growth of yeast strains expressing Hsp82p mutants by increasing speed of doubling in log phase. Cultures were grown overnight in YPD medium at 30°C then diluted to 1 x 10^7 cells per mL. Cultures were grown for 24 hours in YPD medium at both 30°C and 35.5°C taking 600.0nm O.D. readings at 4 hours, 12 hours, and 24 hours. These readings were then charted using the Prism computer graphing program.

3.5 Deletion of *HCH1* is the True Cause of Restoration of Growth in Yeast Strains Expressing the Hsp90 Mutants, Hsp82p^{A587T} or Hsp82p^{G313S}.

We hypothesized that if deletion of *HCH1* was the cause of restoration of growth in the yeast strains expressing the Hsp82p mutants, Hsp82p^{A587T} and Hsp82p^{G313S}, then reintroducing Hch1p to these deletion strains would prevent growth being restored. We introduced a Geneticin(G418)-selectable over-expression plasmid, encoding a C-terminally myc tagged Hch1p, into the iTHisHsp82p, iTHisHsp82p^{A587T} and iTHisHsp82p^{G313S}, co-chaperone knock out strains to determine the effects of reintroducing Hch1p into the *Δhch1* strains. Growth assays showed that the iTHisHsp82p strains were not affected by the over-expression of Hch1p (Figure 3.3A (Armstrong et al. 2012)). Growth of the iTHisHsp82p^{A587T} strains, that over-express Hch1p, was reduced greatly even at 30°C (Figure 3.3A (Armstrong et al. 2012)). As previously shown, when *HCH1* was deleted in the iTHisHsp82p^{A587T} strains at high temperatures growth was restored (Figure 3.3A (Armstrong et al. 2012)). The introduction of the Hch1p over-expression plasmid impaired growth in iTHisHsp82p^{A587T} mutant strains (Figure 3.3A (Armstrong et al. 2012)). Unfortunately we were not able to obtain any Hsp82p^{G313S} G418-resistant clones when the G418-selectable Hch1p over-expression plasmid was transformed into yeast iTHisHsp82p^{G313S} strains. This potentially indicated that yeast cells expressing the Hsp82p^{G313S} mutant are more sensitive to substrate burden than those expressing the Hsp82p^{A587T} mutant, however, more experiments would be necessary to confirm this. We concluded that as reintroduction of Hch1p prevented *Δhch1* restoring growth to iTHisHsp82p^{A587T}, at the restrictive temperature, deletion of *HCH1* is the true cause of restoration of growth in these mutant strains (Armstrong et al. 2012). Over-expression of Hch1p caused the growth of iTHisHsp82p^{A587T} strains to

decrease greatly, even at the permissive temperature, allowing us to also conclude that over-expression of *HCH1* is detrimental to growth of yeast expressing the Hsp82p^{A587T} mutant (Figure 3.3A (Armstrong et al. 2012)).

In order to rule out that this decrease in growth in yeast expressing Hsp82p^{A587T} was due to a decrease in levels of Hsp90 when Hch1p was reintroduced to these strains we performed western blots against His-tagged Hsp82p. We also performed western blots against myc-tagged co-chaperones to determine Hch1p was being over expressed in these strains. Protein levels were examined in the iTHisHsp82p and iTHisHsp82p^{A587T} strains and revealed that Hsp82p was expressed at comparable levels at 30°C and showed that Hch1p was being over expressed in these strains (Figure 3.3B (Armstrong et al. 2012)). We concluded that the level of Hsp90 in the G418-selectable strains was not responsible for the results of reintroducing Hch1p to iTHisHsp82p^{A587T} (Armstrong et al. 2012).

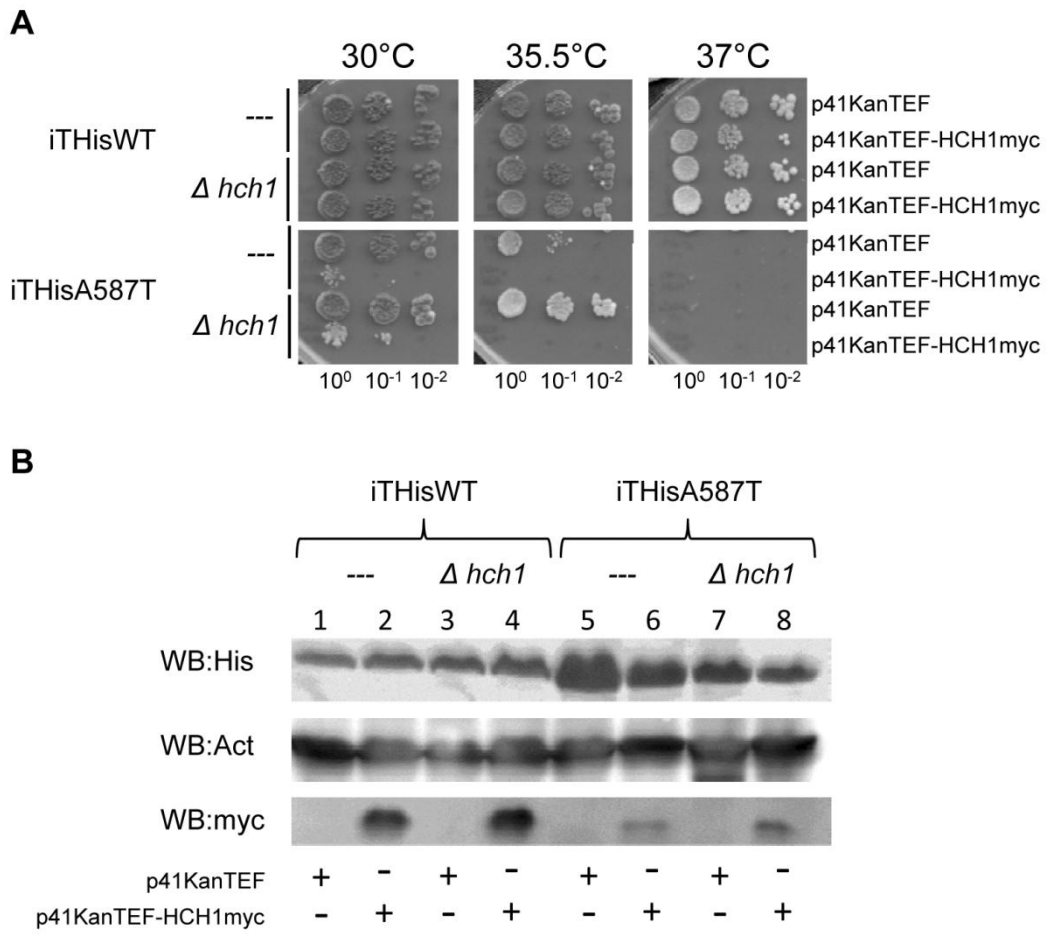

Figure 3.3: Introduction of a G418-selectable plasmid that over-expression of C-terminally myc-tagged Hch1p impairs growth of yeast strains expressing the temperature-sensitive Hsp90 mutant Hsp82p^{A587T} and does not affect yeast expressing Hsp82p. (A) Cultures were grown in YPD+200mgG418/L medium overnight at 30°C. Cultures were diluted to 1 x 10^8 cells per mL and 10-fold serial dilutions were prepared. 5µl of each serial dilution was spotted on YPD+200 mg G418/L medium agar plates and grown for 2 days at 30°C, 35.5°C, and 37°C. (B) Western blot analysis of yeast strains examined in figure 3.3A with anti-His-tag, anti-myc-tag, and anti-actin antibodies. Levels of mycHch1p (lanes 2, 4, 6, and 8) were examined in yeast strains expressing HisHsp82p (lanes 1-4) or HisHsp82p^{A587T} (lanes 5-8) with *HCH1* deletion (lanes 3, 4, 7, and 8) or without *HCH1* deletion (lanes 1, 2, 5, and 6). Figure published by Armstrong *et al.* (Armstrong et al. 2012).

3.6 Deletion of *HCH1* Increases Resistance to Hsp90 Inhibitor Drugs in Yeast Expressing Hsp82p^{A587T} or Hsp82p^{G313S}.

The mutants, Hsp82p^{A587T} and Hsp82p^{G313S}, have been shown to be hypersensitive to geldanamycin, an Hsp90 inhibitor drug (Piper et al. 2003). As deletion of *HCH1* restored growth of yeast expressing Hsp82p^{A587T} or Hsp82p^{G313S} under heat stress, we hypothesized deletion of *HCH1* could also restore normal resistance to Hsp90 inhibitors. We initially used geldanamycin and its derivative 17-AAG in our growth assays but their poor solubility made casting plates with high drug concentrations a challenge. To overcome this we used a new Hsp90 inhibitor NVP-AUY922 (Eccles et al. 2008; Jensen et al. 2008; Stuhmer et al. 2008). Initial growth assays on the iTHisHsp82p, iTHisHsp82p^{A587T}, and iTHisHsp82p^{G313S} co-chaperone knock out strains showed that deletion of *HCH1* but not *AHA1* increased the resistance of iTHisHsp82p^{A587T} and iTHisHsp82p^{G313S} to 17-AAG up to 300µM (Figure 3.4A). Growth assays on the iTHisHsp82p, iTHisHsp82p^{A587T}, and iTHisHsp82p^{G313S} co-chaperone knock out strains showed that deletion of *HCH1* but not *AHA1* increased the resistance of iTHisHsp82p^{A587T} and iTHisHsp82p^{G313S} to NVP-AUY922 up to 50µM (Figure 3.4B (Armstrong et al. 2012)). Surprisingly, even wild-type cells begin to display growth arrest at 200µM NVP-AUY922 but gain resistance to NVP-AUY922 at 200µM when *HCH1* is deleted (Figure 3.4B (Armstrong et al. 2012)). Growth assays on the iTHisHsp82p and iTHisHsp82p^{A587T} G418-selectable strains showed that deletion of *HCH1* increased resistance of iTHisHsp82p^{A587T} strains to NVP-AUY922, and reintroduction of Hch1p to the *Δhch1* strain reverses this increased resistance in these mutants (Figure 3.4C (Armstrong et al. 2012)). As the mutant iTHisHsp82p^{A587T} strains showed a severe growth defect both at high temperatures and in the presence of Hsp90 inhibitor drugs, at permissive temperatures,

we concluded these results support our initial hypothesis for the cause of the *ts* growth defect. We hypothesized the cause of the temperature-sensitive growth defect of our mutant strains is that mutant Hsp82p is unable to deal with the substrate burden on the Hsp90 cycle when exposed to cellular stress (Figure 3.5). When cells contain wild-type Hsp90 they are capable of dealing with greater substrate burden during heat stress, and are able to cope with the decrease in functional Hsp90 caused by Hsp90 inhibitor drugs. When cells contain only mutant Hsp90 they are not able to deal with substrate burden during heat stress and they are not able to cope with the decrease in functional Hsp90 caused by Hsp90-inhibitors. In the presence of Hsp90 inhibitor drugs, even at permissive temperatures, the available pool of mutant Hsp90 capable of functioning to deal with substrate proteins is decreased greatly.

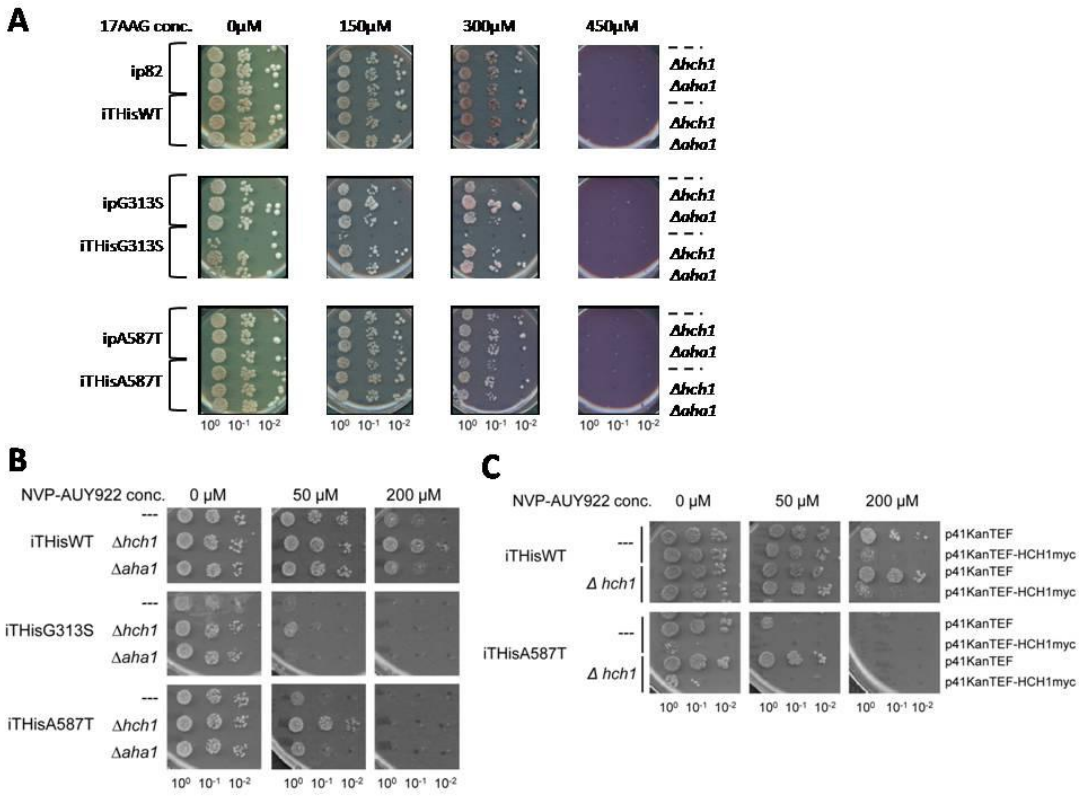

Figure 3.4: Deletion of *HCH1* confers resistance of yeast strains expressing Hsp82p, Hsp82p^{A587T}, or Hsp82p^{G313S}, to specific Hsp90 inhibitor drugs. (A) Yeast strains obtained from Dr. Susan Lindquist (MIT), and those we constructed (iT) were examined in the presence of the Hsp90 inhibitor 17AAG. Cultures grown in YPD medium overnight at 30°C were diluted to 1 x 10^8 cells per mL and 10-fold serial dilutions were prepared. 5µl of each serial dilution was spotted on YPD+17AAG agar plates at 0µM, 150µM, 300µM, and 450µM 17AAG and grown for 2 days at 30°C. (B) Yeast strains (iTHis) were examined in the presence of NVP-AUY922. Cultures grown in YPD medium overnight at 30°C were diluted to 1 x 10^8 cells per mL and 10-fold serial dilutions were prepared. 5µl of each serial dilution was spotted on YPD+NVP-AUY922 agar plates at 0µM, 50µM, 200µM NVP-AUY922 and grown for 2 days at 30°C. (C) The G418-selectable Hch1p reintroduction strains were examined in the presence of NVP-AUY922. Cultures grown in YPD+200mg G418/L medium overnight at 30°C were diluted to 1 x 10^8 cells per mL and 10-fold serial dilutions were prepared. 5µl of each serial dilution was spotted on YPD+ G418+NVP-AUY922 agar plates at 0µM, 50µM, 200µM NVP-AUY922 and grown for 2 days at 30°C. Figure 3.4B and 3.4C publish by Armstrong *et al.* (Armstrong et al. 2012).

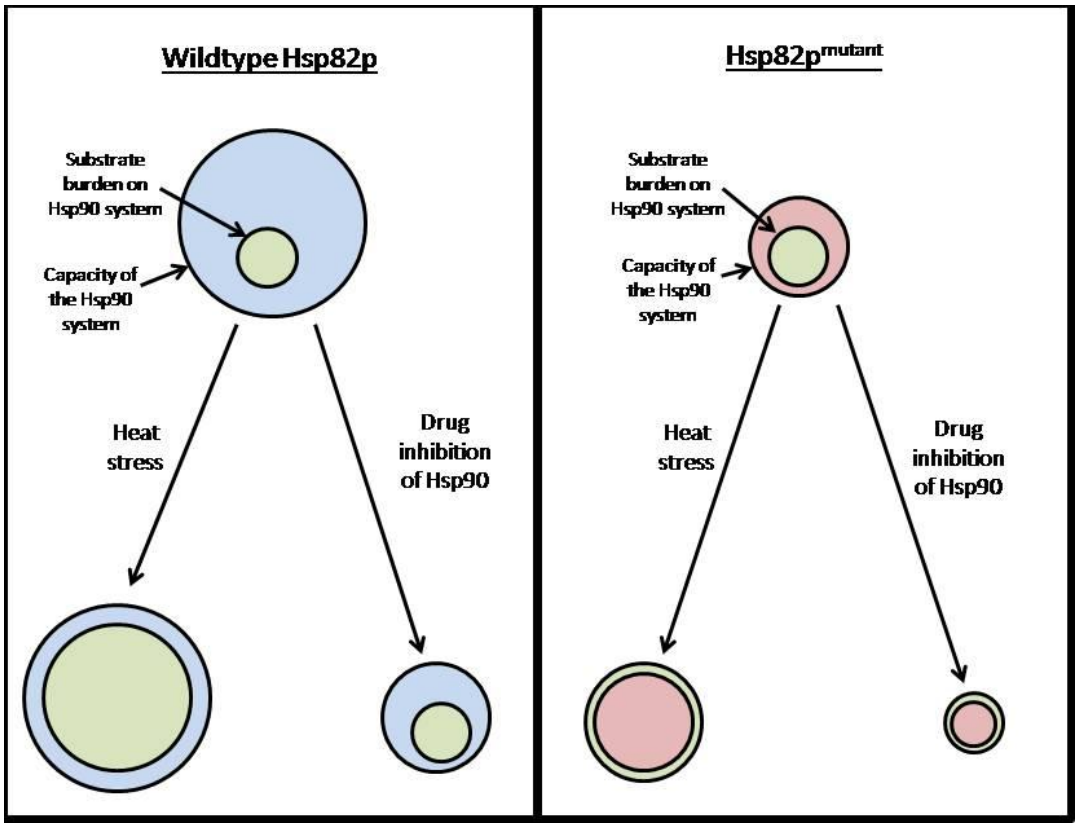

Figure 3.5: A simplified model of the substrate burden on the Hsp90 system during heat-stress and drug inhibition. Yeast containing wild-type Hsp82p have a greater capacity for the Hsp90 system to function than yeast containing *ts* mutant forms of Hsp82p. The amount of substrate burden is increased during heat stress, and the amount of functional Hsp82p is decreased in the presence of Hsp90-inhibitor drugs.

3.7 Hsp82p^{V391E} Mutant Does Not Replicate *Δhch1* Phenotype.

In attempts to phenocopy this *Δhch1* rescue without deleting *HCH1* we introduced another Hsp90 mutant, Hsp82p^{V391E}. Hsp82p^{V391E} blocks binding of Hch1p and Aha1p to Hsp90M. We hypothesized that introduction of this mutation would replicate the rescue of growth found in the *Δhch1* strains however mutant strains expressing Hsp82p$^{A587T\text{-}V391E}$ or Hsp82p$^{G313S\text{-}V391E}$ were not viable (Figure 3.6). This led us to hypothesize that another possible co-chaperone binds this site of Hsp90M and is essential for the progression of the Hsp90 cycle in these mutant strains, or that mutant strains are no longer capable of supporting substrate burden with the addition of the Hsp82p^{V391E} mutation. This hypothesis remains untested.

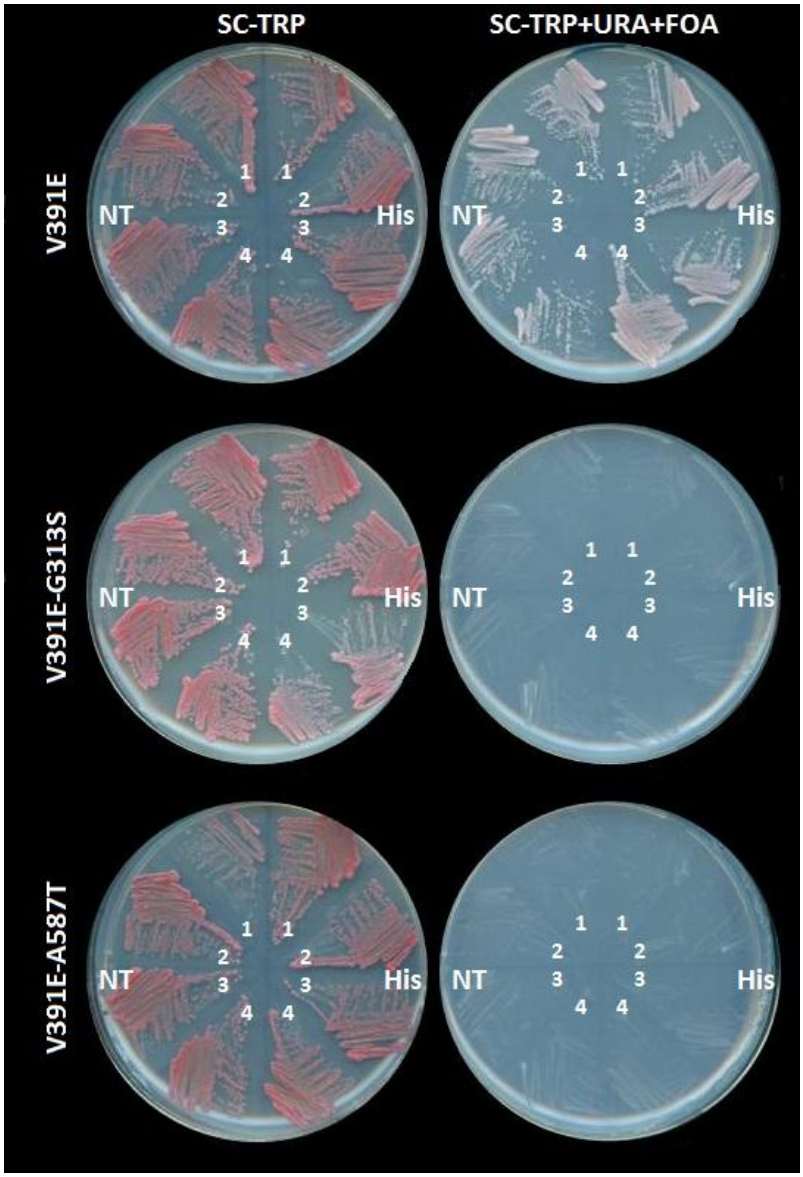

Figure 3.6: A second Hsp90 mutation, Hsp82p^{V319E}, was introduced into Hsp82p, Hsp82p^{G313S} and Hsp82p^{A587T} within the p404GPD plasmid. Following plasmid shuffling of p404GPD plasmids containing Hsp82p^{V391E}, Hsp82p$^{G313S\text{-}V391E}$ and Hsp82p$^{A587T\text{-}V391E}$ into yeast cells, cultures were grown on SC-TRP medium agar plates at 30°C for 2 days to select for tryptophan autotrophy. Clones of untagged (NT) strains and His-tagged (His) strains were then streaked on SC-TRP plates as a control and SC-TRP+URA+1g 5FOA/L medium plates to shuffle out the URA3-selectable plasmid containing the wild-type Hsc82p ensuring cells were expressing Hsp82p^{V391E}, Hsp82p$^{G313S\text{-}V391E}$ or Hsp82p$^{A587T\text{-}V391E}$ as their sole source of Hsp90.

CHAPTER FOUR

Results – The Co-chaperone Hch1p Acts Differently from its Homologue, Aha1p, and Regulates Growth of Yeast Expressing the Temperature-sensitive Hsp90 Mutant, Hsp82p^{E381K}

4.1 *HCH1,* but not *AHA1,* Over-expression Restores Growth to Yeast Expressing the Hsp90 Mutant, Hsp82p^{E381K}.

Previous work has shown that induction of a plasmid that over-expresses the Hsp90 co-chaperone Hch1p rescues the *ts* growth phenotype of yeast expressing the Hsp90 mutant Hsp82p^{E381K} up to 37°C (Nathan et al. 1999). As the N-terminal domain of Aha1p (Aha1p$^{1-156}$) is homologous to Hch1p (Figure 1.4), we hypothesized that over-expression of Aha1p$^{1-156}$ would also be capable of rescuing the *ts* growth phenotype of yeast expressing Hsp82p^{E381K}. In order to test this hypothesis we transformed galactose(GAL)-inducible *AHA1, HCH1,* and *AHA1$^{1-156}$* over-expression plasmids into our iTHisHsp82p, iTHisHsp82p^{E381K}, iTNTHsp82p and iTNTHsp82p^{E381K} strains. Growth assays were performed to determine the growth properties of the iTHisHsp82p and iTHisHsp82p^{E381K} co-chaperone over-expression strains. We were able to replicate previous results showing restoration of growth of yeast expressing Hsp82p^{E381K} by over-expression of Hch1p. At 30°C the iTHisHsp82p^{E381K} co-chaperone over-expression strains already displayed severe growth defects (Figure 4.1A). When *HCH1* over-expression was induced on the galactose plates at 30°C, and even more clearly at 37°C, the growth of iTHisHsp82p^{E381K} strains was restored (Figure 4.1A). Although *HCH1* is homologous to the N-terminus of *AHA1* neither *AHA1* nor *AHA1$^{1-156}$* were capable of restoring growth rescue in iTHisHsp82p^{E381K} (Figure 4.1A). In order to rule out the possibility that restoration of growth was due to a decrease in Hsp82 we performed western blots (Figure 4.1B). While performing experiments to determine if over-expression of Hch1 was causing a decrease in Hsp82 expression we determined that there had been a mix up in the strains and we were actually using a myc-tagged E381K series of yeast strains rather than a Histidine tag series by accident (Figure 4.1B). New experiments using the

His-tagged E381K series demonstrated that these his-tagged E381K strains didn't grow the same on glucose, however, they display similar restoration of growth (data not shown). We believe this difference in growth restoration between strains expressing the myc-tagged and His-tagged E381K strains is due to the presence of the His-tag on E381K.

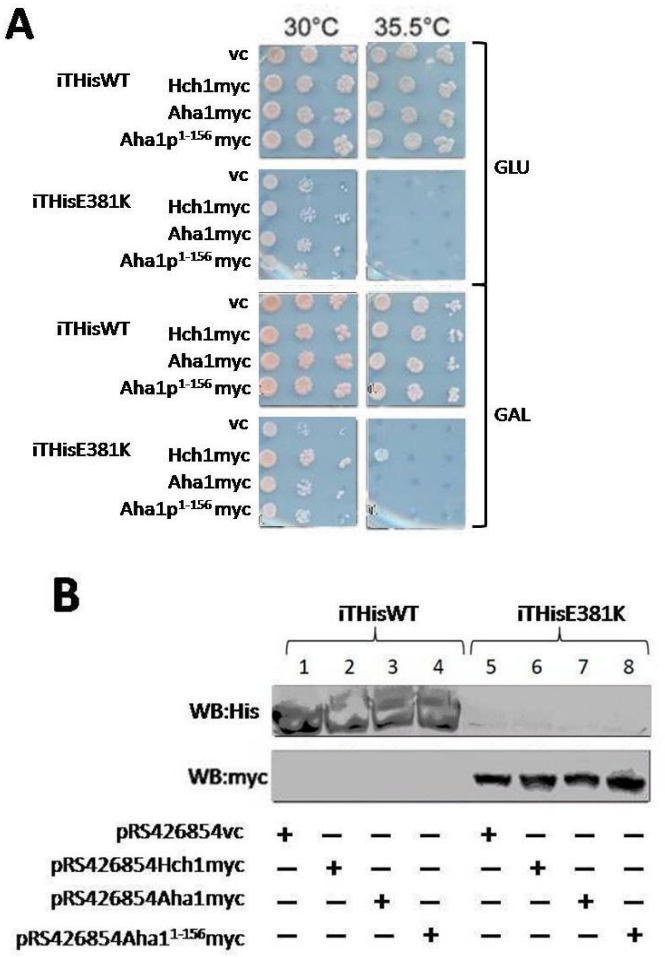

Figure 4.1: Over-expression Hch1p but not its homologue Aha1p restores growth at high temperature in the *S. cerevisiae* strains expressing Hsp82p^{E381K} as their sole source of Hsp90. Cultures were grown in SC-TRP-URA+RAF medium overnight at 30°C. Cultures were diluted to 1 x 10^8 cells per mL and 10-fold serial dilutions were prepared. Cultures were also diluted to 2 x 10^8 cells per mL and grown in SC-TRP-URA+GAL medium overnight at 30°C for use in western blot experiments the following day. 5µl of each serial dilution were spotted on SC-TRP-URA+GLU or SC-TRP-URA+GAL medium agar plates. (A) Viability of the yeast strains iTHisHsp82^{WT} and iTHisHsp82p^{E381K} containing a GAL-driven over-expression of Hch1p, Aha1p, or Aha1p$^{1-156}$, grown for 2 days at 30°C and 35.5°C. (B) Western blot analysis of yeast strains with anti-His-tag and anti-myc-tag antibodies. Levels of Hsp82p were examined at 30°C in yeast strains expressing HisHsp82p (lanes 1-4) or mycHsp82p^{E381K} (lanes 5-8) with over-expression of Hch1p (lanes 2 and 6), Aha1p (lanes 3 and 7) or Aha1p$^{1-156}$ (lanes 4 and 8).

4.2 *HCH1* Knock Out is Lethal in Cells Expressing the Temperature-sensitive Hsp90 Mutant, Hsp82p^{E381K}.

As *HCH1* over-expression clearly rescues yeast expressing Hsp82p^{E381K} as their sole source of Hsp90, we hypothesized that deletion of *HCH1* in these strains would worsen their growth defects. Plasmid shuffling was used to introduce the NTHsp82p^{E381K} or mycHsp82p^{E381K} mutant into the *HCH1* deletion strain, however, these deletion strains were no longer viable in cells containing the NTHsp82p^{E381K} or mycHsp82p^{E381K} mutant as their sole source of Hsp90 (Figure 4.2A). As the *Δaha1* strains did survive this plasmid shuffling, we were able to conclude that NTHsp82p^{E381K} and mycHsp82p^{E381K} requires Hch1p to function but not Aha1p. We were able to replicate results demonstrating Hsp82p^{E381K} still requires Hch1p whether untagged, myc-tagged (Figure 4.2A), or histidine-tagged (Figure 4.2B). We concluded that more experiments will be necessary to determine the relationship between Hsp82p^{E381K} and its co-chaperones Hch1p and Aha1p, however, it is clear that Hsp82p^{E381K} requires Hch1p but not its homologue Aha1p to function *in vivo*.

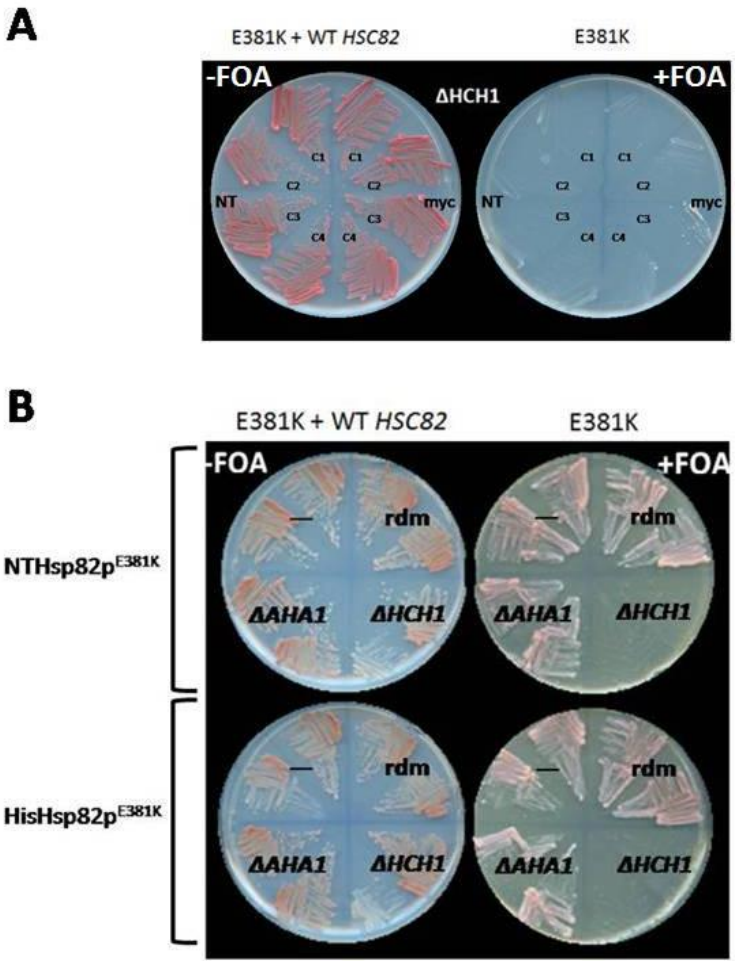

Figure 4.2: Deletion of *HCH1*, but not its homologue *AHA1*, causes *S. cerevisiae* strains expressing Hsp82p^{E381K} as their sole source of Hsp90 to no longer be viable. Following plasmid shuffling of p404GPD plasmids containing *HSP82*^{E381K} into yeast cells, cultures were grown on SC-TRP medium agar plates at 30°C for 2 days to select for tryptophan autotrophy. Clones of myc-tagged (myc) strains, untagged (NT) strains and His-tagged (His) strains were then streaked on SC-TRP plates as a control and SC-TRP+URA+1g 5FOA/L plates to shuffle out the URA3-selectable plasmid containing the wild-type Hsc82p ensuring cells were expressing Hsp82p^{E381K} as their sole source of Hsp90. (A) Viability of strains expressing NTHsp82p^{E381K} or mycHsp82p^{E381K} as their sole source of Hsp90 when *HCH1* is deleted. (B) Viability of strains expressing NTHsp82p^{E381K} or HisHsp82p^{E381K} as their sole source of Hsp90 when *HCH1* or *AHA1* is deleted.

CHAPTER FIVE

Discussion

5.1 The Importance of Studying Hsp90 and the Hsp90 Pathway.

Hsp90 is of significant importance in clinical studies as it facilitates the activation of hundreds of client proteins, many of which are involved in diseases including, but not limited to, neurodegeneration, dementia, type 2 diabetes, cystic fibrosis, and cancer (Samuels et al. 1975; Breitner et al. 1986; Goate et al. 1991; Perutz 1999; Macario et al. 2000; Hull et al. 2004; Whitesell et al. 2005; Chiti et al. 2006; Morimoto 2008; Hahn 2009; Aguzzi et al. 2010; Hartl et al. 2011). Hsp90 inhibitor drugs are capable of targeting multiple signalling pathways implicated in the progression of cancer and have shown promising results in clinical cancer therapeutic studies (Hao et al. 2010; Pimienta et al. 2011; Centenera et al. 2012). More recently, inhibitor drugs targeting Hsp90 co-chaperone proteins have overcome many of the undesirable side effects of Hsp90 inhibitor drugs and have demonstrated the importance of understanding the relationship between Hsp90 and its co-chaperones within the Hsp90 cycle (Pimienta et al. 2011). Specifically, the co-chaperone Aha1p is of importance when studying the Hsp90 pathway due to its involvement in disease (Holmes et al. 2008). We have demonstrated that the Aha1p homologue Hch1p is capable of either assisting or hindering the growth of specific mutants of Hsp90 in a yeast model system, and Hch1p also regulates the sensitivity of Hsp90 to Hsp90-inhibitor drugs. We have therefore concluded that it is essential to determine the relationship between Hch1p and Aha1p, and their involvement in the Hsp90 cycle.

5.2 The Co-chaperone Hch1p is Detrimental to the Growth of Yeast Expressing Hsp90 Mutant Hsp82p^{A587T} or Hsp82p^{G313S}.

As Hch1p and Aha1p are considered homologous we hypothesized deletion of either of these co-chaperones would have the same effect. Interestingly, deletion of *HCH1*, but not its homologue *AHA1*, restored growth to yeast expressing the Hsp90 mutants, Hsp82p^{A587T} or Hsp82p^{G313S} at restrictive temperatures (Armstrong et al. 2012). We were able to rule out the possibility that this restoration was due to a competition between Hch1p and Aha1p for binding at a specific site on Hsp90M (Figure 3.1A), or that deletion of this co-chaperone within these mutant strains was causing an upregulation of Hsp90 (Figure 3.1C (Armstrong et al. 2012)). We demonstrated that *HCH1* deletion, and not some spurious genetic alteration, is responsible for this restoration of growth as reintroduction of Hch1p into the *Δhch1* strains exacerbated the growth defect of yeast expressing Hsp82p^{A587T}. Reintroduction of Hch1p even increased the sensitivity of these mutant strains to heat stress, as strains grew less so at the permissive temperature when Hch1p was over expressed (Figure 3.3 (Armstrong et al. 2012)). We also believe that cells expressing Hsp82p^{A587T} or Hsp82p^{G313S} grow slower in log phase than wild-type cells which relates to previous research showing that yeast containing Hsp82p^{G313S} do not have a defined G1 peak and are largely arrested as 2N cells (McClellan et al. 2007). As deletion of *HCH1* restores the growth of cells containing Hsp82p^{G313S}, this suggests that Hch1p association with mutant Hsp82p^{G313S} is affecting the ability of these yeast cells to progress through the cell cycle. However, more research is necessary as experiments have not been done to examine cell cycle arrest in cells expressing Hsp82p^{A587T} and it would be interesting to see if mutant strains are still arrested when *HCH1* is deleted. I expect that when *HCH1* is deleted in cells containing Hsp82p^{A587T} or

Hsp82p^{G313S}, we would see a more defined G1 peak and fewer cells would be arrested as 2N cells.

We attempted to phenocopy deletion results without deleting *HCH1* or *AHA1* by introducing the mutant Hsp82p^{V391E}, which prevents binding at the site of Hsp90M where Hch1p and Aha1p are thought to bind. Surprisingly, introducing this second mutation, Hsp82p^{V391E}, into the Hsp82p of the iTHisHsp82p^{A587T} and iTHisHsp82p^{G313S} strains resulted in their inability to support viability in yeast. As iTHisHsp82p^{V391E} cells were viable, this suggested that Hch1p could be competing for binding Hsp90M with another unknown co-chaperone protein essential for growth of cells expressing Hsp82p^{A587T} and Hsp82p^{G313S}. Interestingly, previous studies have shown that strains expressing Hsp82p^{G313S} require Sti1p in order to maintain viability, and we believe this is also true for yeast expressing Hsp82p^{A587T} although this has never been tested (Chang et al. 1997). Over-expression of the co-chaperone Sti1p in yeast expressing Hsp82p^{A587T} or Hsp82p^{G313S} has been shown to restore growth in these *ts* strains up to 36°C (Chang et al. 1997). Sti1p is known to bind the MEEVD domain on the C-terminus of Hsp90 to deliver client proteins from Hsp70 to Hsp90, and has also very recently been shown to interact with the Hsp90 middle domain (Schmid et al. 2012). Interestingly, we found that yeast expressing Hsp82p^{V391E} are very sensitive to Hsp90 inhibitor drugs (data not shown) and previous work by Piper *et al.* showed that Sti1p knock out yeast are also more sensitive to Hsp90 inhibitor drugs (Piper et al. 2003). Sti1p could therefore, potentially be the co-chaperone competing for binding with Hch1p at the site of Hsp90M in strains expressing Hsp82p^{A587T} or Hsp82p^{G313S}. Immunoprecipitation experiments could help in elucidating the relationships between these two co-chaperone proteins with each other and the Hsp90 mutants Hsp82p^{A587T} and

Hsp82p^{G313S}. I expect that increasing amounts of Sti1p would be pulled down in complex with Hsp90M when *HCH1* is deleted if Sti1p is competing for binding with Hch1p at Hsp90M. It is important to remember there are many co-chaperone proteins currently described and hundreds of client proteins known to interact with Hsp90 in yeast therefore, immunoprecipitation could potentially indicate other proteins in complex with Hsp90M when *HCH1* is deleted (Mimnaugh et al. 2006; Echeverria et al. 2011). Mass-spectrometry would allow us to determine what these proteins are.

5.3 Co-chaperone Hch1p Regulates Sensitivity of Hsp90 to Hsp90-Inhibitor Drugs.

Excitingly, not only is knock out of *HCH1* capable of restoring growth of yeast expressing Hsp82p^{A587T} or Hsp82p^{G313S} during heat stress, it also causes these mutant strains to display increased resistance to Hsp90 inhibitor drugs at permissive temperatures (Armstrong et al. 2012). Surprisingly, even wild-type yeast strains gain resistance to the Hsp90 inhibitor NVP-AUY922 when *HCH1* is deleted (Armstrong et al. 2012). Reintroduction of Hch1p exacerbates the growth defect of cells expressing Hsp82p^{A587T} or Hsp82p^{G313S} even at the permissive temperature, and over-expression of Hch1p confers hypersensitivity to Hsp90 inhibitor drugs (Armstrong et al. 2012). This allows us to conclude that Hch1p regulates the sensitivity of Hsp90 to Hsp90 inhibitor drugs, and is detrimental to the growth of yeast expressing Hsp90 mutants Hsp82p^{A587T} or Hsp82p^{G313S} (Armstrong et al. 2012). Unfortunately, an undesirable side-effect of these nucleotide pocket Hsp90 inhibitors is the transcriptional upregulation of Hsp27 and Hsp70, which have antiapoptotic effects (Pimienta et al. 2011). This unfavourable characteristic is overcome by the introduction of the Hsp90 cycle inhibitor 1,6-dimethyl-3-propylpyrimido[5,4-e][1,2,4]triazine-5,7-dione, more simply known as C9 (Pimienta et

al. 2011). Unlike the other Hsp90 inhibitors described, C9 does not bind the nucleotide pocket of Hsp90, it instead prevents the interaction of Hsp90 with its co-chaperone Sti1p by binding directly to the TPR2A domain of Sti1p, thus preventing the transfer of client proteins from Hsp70 to Hsp90 (Pimienta et al. 2011). C9 induces degradation of Hsp90 clients and does not cause the undesirable upregulation of Hsp27 or Hsp70 (Pimienta et al. 2011). Exciting characteristics of C9 include its combined affects with either 17AAG or NVP-AUY922, which maintains the positive effects of both inhibitors but prevents the upregulation of Hsp27 and Hsp70 (Pimienta et al. 2011). It would be interesting to examine the affects of C9 on growth of yeast expressing Hsp82p^{G313S} or Hsp82p^{A587T} as this drug prevents the binding of Sti1p to the MEEVD of Hsp90C and as previously mentioned, Sti1p over-expression restores growth in these mutant cell lines. I expect that if the growth defect of cells expressing Hsp82p^{A587T} or Hsp82p^{G313S} is due to Hch1p competing for binding with Sti1p at the site on Hsp90M then deletion of *HCH1* would still be capable of restoring growth of these strains in the presence of C9 as Sti1p binding to Hsp90M is not affected by C9.

5.4 The Hsp90 Co-chaperone Hch1p Functions Differently From its Homologue Aha1p.

We were able to replicate results showing that induction of a plasmid that over-expresses Hch1p restores the growth of yeast expressing the *ts* Hsp90 mutant Hsp82p^{E381K} up to 37°C. We demonstrated that Hch1p is not only required for proper growth in strains expressing Hsp82p^{E381K}, it is actually essential for their survival as deletion of *HCH1* causes strains expressing Hsp82p^{E381K} to lose viability at any temperature. Interestingly, although Hch1p is homologous to the N-terminus of Aha1p

(Aha1p$^{1-156}$), Aha1p is not required for growth of yeast expressing Hsp82p^{E381K}. Although these two co-chaperones are considered homologous we have shown that their activity varies. To determine what it is that makes these co-chaperones different we are currently examining various domain swaps in which segments of *HCH1* are placed into *AHA1*$^{1-156}$ in order to determine which specific amino acid sequences of *HCH1* allow it to restore growth of yeast expressing Hsp82p^{E381K} while the homologous Aha1p$^{1-156}$ is not capable of this restoration. We expect that addition of the specific segment of Hch1p, that allows it to restore growth to yeast expressing Hsp82p^{E381K}, into Aha1p$^{1-156}$ will allow Aha1p$^{1-156}$ to also restore growth in these cells. We also expect addition of this segment of Hch1p into Aha1p$^{1-156}$ will be detrimental to growth of yeast expressing Hsp82p^{A587T} or Hsp82p^{G313S} in the presence of heat-stress and Hsp90-inhibitor drugs.

5.5 Future Directions and Summary.

Preliminary subunit complementation experiments were performed to examine the effects of complementing one Hsp90 mutant with another. We wanted to know if the formation of heterodimers of two different Hsp82p mutants would cause a restoration of growth. We hypothesized heterodimers of the ATPase dead, yet otherwise wild-type, Hsp82p^{D79N}, with Hsp82p^{A587T} or Hsp82p^{G313S} would restore growth in the iTHisHsp82p^{A587T} and iTHisHsp82p^{G313S} strains. We assumed the introduction of Hsp82p^{D79N} would prevent the potential improper conformational changes caused by Hsp82p^{A587T} or Hsp82p^{G313S} mutations, or allow proper binding of co-chaperone proteins. However, co-expression of Hsp82p^{D79N} with Hsp82p^{A587T} or Hsp82p^{G313S} in yeast did not demonstrate any striking results therefore we did not pursue these experiments further. We also did not verify the formation of heterodimers in these experiments therefore

more experiments will be needed before conclusions can be made on these results. To determine if heterodimers are forming we could use a different epitope tag for Hsp82p^{D79N} than for Hsp82p^{A587T} and Hsp82p^{G313S}. In future experiments, it would be interesting to see if heterodimers of Hsp82p^{E381K} with Hsp82p^{A587T} or Hsp82p^{G313S} alleviate the stress induced growth defect observed in yeast expressing these individual Hsp90 mutants, as over-expression of Hch1p restores growth of iTHisHsp82p^{E381K}, while deletion of *HCH1* restores growth of iTHisHsp82p^{A587T} and iTHisHsp82p^{G313S}.

All of the evidence described so far points towards Hch1p functioning closely to Sti1p in the Hsp90 cycle, and not its homologue Aha1p (Figure 1.2). It appears that the co-chaperones Hch1p and Sti1p have opposite effects in yeast expressing Hsp82p^{A587T} and Hsp82p^{G313S} and that Hch1p likely competes with Sti1p for binding at the middle domain of Hsp90. Sti1p is essential for yeast expressing Hsp82p^{G313S} and likely Hsp82p^{A587T} and confers resistance of wild-type, Hsp82p^{A587T} and Hsp82p^{G313S} expressing strains to Hsp90 inhibitor drugs (Chang et al. 1997; Piper et al. 2003). Hch1p exacerbates the growth defect of cells expressing Hsp82p^{A587T} and Hsp82p^{G313S} and confers hypersensitivity of wild-type, Hsp82p^{A587T} and Hsp82p^{G313S} strains to Hsp90 inhibitor drugs. We therefore believe that Hch1p acts early on in the Hsp90 cycle at the stage of client engagement, however, it is still not clear exactly what Hch1p does within the Hsp90 cycle.

We have determined that the specific expression of the Hsp90 co-chaperone Hch1p, but not its homologue Aha1p, is able to alleviate certain growth defects in specific *S. cerevisiae* Hsp90 mutant strains (Armstrong et al. 2012). We propose that the observed temperature-sensitive phenotype of yeast expressing the Hsp90 mutant Hsp82p^{A587T}, Hsp82p^{G313S}, or Hsp82p^{E381K}, is due to an inability of mutant Hsp90 to deal

with an increase in substrate burden on the Hsp90 system, and that the capacity for these mutants to process substrates under conditions of high burden is altered by Hch1p, but not its homologue Aha1p. There is however much that remains to be elucidated about the Hsp90 cycle and its relationship with co-chaperone and client proteins.

CHAPTER SIX

References

Aguzzi, A. and T. O'Connor (2010). "Protein aggregation diseases: pathogenicity and therapeutic perspectives." Nat Rev Drug Discov **9**(3): 237-248.
Ali, M. M., S. M. Roe, et al. (2006). "Crystal structure of an Hsp90-nucleotide-p23/Sba1 closed chaperone complex." Nature **440**(7087): 1013-1017.
Anfinsen, C. B. (1973). "Principles that govern the folding of protein chains." Science **181**(4096): 223-230.
Armstrong, H., A. Wolmarans, et al. (2012). "The Co-Chaperone Hch1 Regulates Hsp90 Function Differently than Its Homologue Aha1 and Confers Sensitivity to Yeast to the Hsp90 Inhibitor NVP-AUY922." PLoS One **7**(11): e49322.
Ban, C., M. Junop, et al. (1999). "Transformation of MutL by ATP binding and hydrolysis: a switch in DNA mismatch repair." Cell **97**(1): 85-97.
Ban, C. and W. Yang (1998). "Crystal structure and ATPase activity of MutL: implications for DNA repair and mutagenesis." Cell **95**(4): 541-552.
Banerji, U., A. O'Donnell, et al. (2005). "Phase I pharmacokinetic and pharmacodynamic study of 17-allylamino, 17-demethoxygeldanamycin in patients with advanced malignancies." J Clin Oncol **23**(18): 4152-4161.
Bohen, S. P. (1998). "Genetic and biochemical analysis of p23 and ansamycin antibiotics in the function of Hsp90-dependent signaling proteins." Mol Cell Biol **18**(6): 3330-3339.
Bracher, A. and F. U. Hartl (2006). "Hsp90 structure: when two ends meet." Nat Struct Mol Biol **13**(6): 478-480.
Breitner, J. C., M. F. Folstein, et al. (1986). "Familial aggregation in Alzheimer dementia--I. A model for the age-dependent expression of an autosomal dominant gene." J Psychiatr Res **20**(1): 31-43.
Breitner, J. C., E. A. Murphy, et al. (1986). "Familial aggregation in Alzheimer dementia--II. Clinical genetic implications of age-dependent onset." J Psychiatr Res **20**(1): 45-55.
Brough, P. A., W. Aherne, et al. (2008). "4,5-diarylisoxazole Hsp90 chaperone inhibitors: potential therapeutic agents for the treatment of cancer." J Med Chem **51**(2): 196-218.
Buchanan, G., C. Ricciardelli, et al. (2007). "Control of androgen receptor signaling in prostate cancer by the cochaperone small glutamine rich tetratricopeptide repeat containing protein alpha." Cancer Res **67**(20): 10087-10096.
Buchner, J. (1996). "Supervising the fold: functional principles of molecular chaperones." FASEB J **10**(1): 10-19.
Buchner, J. (1999). "Hsp90 & Co. - a holding for folding." Trends Biochem Sci **24**(4): 136-141.
Caplan, A. J. (2003). "What is a co-chaperone?" Cell Stress Chaperones **8**(2): 105-107.
Centenera, M. M., J. L. Gillis, et al. (2012). "Evidence for efficacy of new Hsp90 inhibitors revealed by ex vivo culture of human prostate tumors." Clin Cancer Res **18**(13): 3562-3570.
Chadli, A., I. Bouhouche, et al. (2000). "Dimerization and N-terminal domain proximity underlie the function of the molecular chaperone heat shock protein 90." Proc Natl Acad Sci U S A **97**(23): 12524-12529.
Chang, H. C., D. F. Nathan, et al. (1997). "In vivo analysis of the Hsp90 cochaperone Sti1 (p60)." Mol Cell Biol **17**(1): 318-325.

Cheetham, M. E. and A. J. Caplan (1998). "Structure, function and evolution of DnaJ: conservation and adaptation of chaperone function." Cell Stress Chaperones **3**(1): 28-36.

Chen, S. and D. F. Smith (1998). "Hop as an adaptor in the heat shock protein 70 (Hsp70) and hsp90 chaperone machinery." J Biol Chem **273**(52): 35194-35200.

Chiti, F. and C. M. Dobson (2006). "Protein misfolding, functional amyloid, and human disease." Annu Rev Biochem **75**: 333-366.

Citri, A., D. Harari, et al. (2006). "Hsp90 recognizes a common surface on client kinases." J Biol Chem **281**(20): 14361-14369.

Cox, M. B. and C. A. Miller, 3rd (2003). "Pharmacological and genetic analysis of 90-kDa heat shock isoprotein-aryl hydrocarbon receptor complexes." Mol Pharmacol **64**(6): 1549-1556.

Csermely, P., T. Schnaider, et al. (1998). "The 90-kDa molecular chaperone family: structure, function, and clinical applications. A comprehensive review." Pharmacol Ther **79**(2): 129-168.

Dobson, C. M. and R. J. Ellis (1998). "Protein folding and misfolding inside and outside the cell." EMBO J **17**(18): 5251-5254.

Dollins, D. E., J. J. Warren, et al. (2007). "Structures of GRP94-nucleotide complexes reveal mechanistic differences between the hsp90 chaperones." Mol Cell **28**(1): 41-56.

Dutta, R. and M. Inouye (2000). "GHKL, an emergent ATPase/kinase superfamily." Trends Biochem Sci **25**(1): 24-28.

Eccles, S. A., A. Massey, et al. (2008). "NVP-AUY922: a novel heat shock protein 90 inhibitor active against xenograft tumor growth, angiogenesis, and metastasis." Cancer Res **68**(8): 2850-2860.

Echeverria, P. C., A. Bernthaler, et al. (2011). "An interaction network predicted from public data as a discovery tool: application to the Hsp90 molecular chaperone machine." PLoS One **6**(10): e26044.

Egorin, M. J., E. G. Zuhowski, et al. (2001). "Plasma pharmacokinetics and tissue distribution of 17-(allylamino)-17-demethoxygeldanamycin (NSC 330507) in CD2F1 mice1." Cancer Chemother Pharmacol **47**(4): 291-302.

Ellis, J. (1987). "Proteins as molecular chaperones." Nature **328**(6129): 378-379.

Ellis, R. J. (1996). "Discovery of molecular chaperones." Cell Stress Chaperones **1**(3): 155-160.

Ellis, R. J. (2001). "Macromolecular crowding: obvious but underappreciated." Trends Biochem Sci **26**(10): 597-604.

Ellis, R. J. and S. M. van der Vies (1991). "Molecular chaperones." Annu Rev Biochem **60**: 321-347.

Felts, S. J., B. A. Owen, et al. (2000). "The hsp90-related protein TRAP1 is a mitochondrial protein with distinct functional properties." J Biol Chem **275**(5): 3305-3312.

Gaiser, A. M., A. Kretzschmar, et al. (2010). "Cdc37-Hsp90 complexes are responsive to nucleotide-induced conformational changes and binding of further cofactors." J Biol Chem **285**(52): 40921-40932.

Ge, J., E. Normant, et al. (2006). "Design, synthesis, and biological evaluation of hydroquinone derivatives of 17-amino-17-demethoxygeldanamycin as potent, water-soluble inhibitors of Hsp90." J Med Chem **49**(15): 4606-4615.

Goate, A., M. C. Chartier-Harlin, et al. (1991). "Segregation of a missense mutation in the amyloid precursor protein gene with familial Alzheimer's disease." Nature **349**(6311): 704-706.

Guo, W., P. Reigan, et al. (2005). "Formation of 17-allylamino-demethoxygeldanamycin (17-AAG) hydroquinone by NAD(P)H:quinone oxidoreductase 1: role of 17-AAG hydroquinone in heat shock protein 90 inhibition." Cancer Res **65**(21): 10006-10015.

Hahn, J. S. (2009). "The Hsp90 chaperone machinery: from structure to drug development." BMB Rep **42**(10): 623-630.

Hainzl, O., M. C. Lapina, et al. (2009). "The charged linker region is an important regulator of Hsp90 function." J Biol Chem **284**(34): 22559-22567.

Hao, H., Y. Naomoto, et al. (2010). "HSP90 and its inhibitors." Oncol Rep **23**(6): 1483-1492.

Harst, A., H. Lin, et al. (2005). "Aha1 competes with Hop, p50 and p23 for binding to the molecular chaperone Hsp90 and contributes to kinase and hormone receptor activation." Biochem J **387**(Pt 3): 789-796.

Hartl, F. U. (1996). "Molecular chaperones in cellular protein folding." Nature **381**(6583): 571-579.

Hartl, F. U., A. Bracher, et al. (2011). "Molecular chaperones in protein folding and proteostasis." Nature **475**(7356): 324-332.

Haslbeck, M. (2002). "sHsps and their role in the chaperone network." Cell Mol Life Sci **59**(10): 1649-1657.

Hawle, P., M. Siepmann, et al. (2006). "The middle domain of Hsp90 acts as a discriminator between different types of client proteins." Mol Cell Biol **26**(22): 8385-8395.

Hollingshead, M., M. Alley, et al. (2005). "In vivo antitumor efficacy of 17-DMAG (17-dimethylaminoethylamino-17-demethoxygeldanamycin hydrochloride), a water-soluble geldanamycin derivative." Cancer Chemother Pharmacol **56**(2): 115-125.

Holmes, J. L., S. Y. Sharp, et al. (2008). "Silencing of HSP90 cochaperone AHA1 expression decreases client protein activation and increases cellular sensitivity to the HSP90 inhibitor 17-allylamino-17-demethoxygeldanamycin." Cancer Res **68**(4): 1188-1197.

Hostein, I., D. Robertson, et al. (2001). "Inhibition of signal transduction by the Hsp90 inhibitor 17-allylamino-17-demethoxygeldanamycin results in cytostasis and apoptosis." Cancer Res **61**(10): 4003-4009.

Hull, R. L., G. T. Westermark, et al. (2004). "Islet amyloid: a critical entity in the pathogenesis of type 2 diabetes." J Clin Endocrinol Metab **89**(8): 3629-3643.

Hwang, K., C. D. Scripture, et al. (2006). "Determination of the heat shock protein 90 inhibitor 17-dimethylaminoethylamino-17-demethoxygeldanamycin in plasma by liquid chromatography-electrospray mass spectrometry." J Chromatogr B Analyt Technol Biomed Life Sci **830**(1): 35-40.

Jakob, U., M. Gaestel, et al. (1993). "Small heat shock proteins are molecular chaperones." J Biol Chem **268**(3): 1517-1520.

Jensen, M. R., J. Schoepfer, et al. (2008). "NVP-AUY922: a small molecule HSP90 inhibitor with potent antitumor activity in preclinical breast cancer models." Breast Cancer Res **10**(2): R33.

Johnson, J. L. and C. Brown (2009). "Plasticity of the Hsp90 chaperone machine in divergent eukaryotic organisms." Cell Stress Chaperones **14**(1): 83-94.
Johnson, J. L. and E. A. Craig (1997). "Protein folding in vivo: unraveling complex pathways." Cell **90**(2): 201-204.
Johnson, J. L., A. Halas, et al. (2007). "Nucleotide-dependent interaction of Saccharomyces cerevisiae Hsp90 with the cochaperone proteins Sti1, Cpr6, and Sba1." Mol Cell Biol **27**(2): 768-776.
Johnson, J. L. and D. O. Toft (1995). "Binding of p23 and hsp90 during assembly with the progesterone receptor." Mol Endocrinol **9**(6): 670-678.
Ki, S. W., K. Ishigami, et al. (2000). "Radicicol binds and inhibits mammalian ATP citrate lyase." J Biol Chem **275**(50): 39231-39236.
Koulov, A. V., P. Lapointe, et al. (2010). "Biological and structural basis for Aha1 regulation of Hsp90 ATPase activity in maintaining proteostasis in the human disease cystic fibrosis." Mol Biol Cell **21**(6): 871-884.
Kwon, H. J., M. Yoshida, et al. (1992). "Radicicol, an agent inducing the reversal of transformed phenotypes of src-transformed fibroblasts." Biosci Biotechnol Biochem **56**(3): 538-539.
Kwon, H. J., M. Yoshida, et al. (1997). "Suppression of morphological transformation by radicicol is accompanied by enhanced gelsolin expression." Oncogene **15**(21): 2625-2631.
Lamb, J. R., S. Tugendreich, et al. (1995). "Tetratrico peptide repeat interactions: to TPR or not to TPR?" Trends Biochem Sci **20**(7): 257-259.
Laskey, R. A., B. M. Honda, et al. (1978). "Nucleosomes are assembled by an acidic protein which binds histones and transfers them to DNA." Nature **275**(5679): 416-420.
Lee, C. C., T. W. Lin, et al. (2011). "The hexameric structures of human heat shock protein 90." PLoS One **6**(5): e19961.
Lee, S. and F. T. Tsai (2005). "Molecular chaperones in protein quality control." J Biochem Mol Biol **38**(3): 259-265.
Lindquist, S. and E. A. Craig (1988). "The heat-shock proteins." Annu Rev Genet **22**: 631-677.
Lotz, G. P., H. Lin, et al. (2003). "Aha1 binds to the middle domain of Hsp90, contributes to client protein activation, and stimulates the ATPase activity of the molecular chaperone." J Biol Chem **278**(19): 17228-17235.
Louvion, J. F., R. Warth, et al. (1996). "Two eukaryote-specific regions of Hsp82 are dispensable for its viability and signal transduction functions in yeast." Proc Natl Acad Sci U S A **93**(24): 13937-13942.
Macario, A. J. and E. Conway de Macario (2000). "Stress and molecular chaperones in disease." Int J Clin Lab Res **30**(2): 49-66.
Maki, R. G., L. J. Old, et al. (1990). "Human homologue of murine tumor rejection antigen gp96: 5'-regulatory and coding regions and relationship to stress-induced proteins." Proc Natl Acad Sci U S A **87**(15): 5658-5662.
McClellan, A. J., Y. Xia, et al. (2007). "Diverse cellular functions of the Hsp90 molecular chaperone uncovered using systems approaches." Cell **131**(1): 121-135.
McLaughlin, S. H., H. W. Smith, et al. (2002). "Stimulation of the weak ATPase activity of human hsp90 by a client protein." J Mol Biol **315**(4): 787-798.

Meyer, P., C. Prodromou, et al. (2003). "Structural and functional analysis of the middle segment of hsp90: implications for ATP hydrolysis and client protein and cochaperone interactions." Mol Cell **11**(3): 647-658.
Meyer, P., C. Prodromou, et al. (2004). "Structural basis for recruitment of the ATPase activator Aha1 to the Hsp90 chaperone machinery." EMBO J **23**(3): 511-519.
Millson, S. H., A. W. Truman, et al. (2004). "Investigating the protein-protein interactions of the yeast Hsp90 chaperone system by two-hybrid analysis: potential uses and limitations of this approach." Cell Stress Chaperones **9**(4): 359-368.
Millson, S. H., C. K. Vaughan, et al. (2008). "Chaperone ligand-discrimination by the TPR-domain protein Tah1." Biochem J **413**(2): 261-268.
Mimnaugh, E. G., W. Xu, et al. (2006). "Endoplasmic reticulum vacuolization and valosin-containing protein relocalization result from simultaneous hsp90 inhibition by geldanamycin and proteasome inhibition by velcade." Mol Cancer Res **4**(9): 667-681.
Miyata, Y. (2005). "Hsp90 inhibitor geldanamycin and its derivatives as novel cancer chemotherapeutic agents." Curr Pharm Des **11**(9): 1131-1138.
Morano, K. A. (2007). "New tricks for an old dog: the evolving world of Hsp70." Ann N Y Acad Sci **1113**: 1-14.
Morano, K. A., P. C. Liu, et al. (1998). "Protein chaperones and the heat shock response in Saccharomyces cerevisiae." Curr Opin Microbiol **1**(2): 197-203.
Morimoto, R. I. (2008). "Proteotoxic stress and inducible chaperone networks in neurodegenerative disease and aging." Genes Dev **22**(11): 1427-1438.
Mumberg, D., R. Muller, et al. (1995). "Yeast vectors for the controlled expression of heterologous proteins in different genetic backgrounds." Gene **156**(1): 119-122.
Munster, P. N., A. Basso, et al. (2001). "Modulation of Hsp90 function by ansamycins sensitizes breast cancer cells to chemotherapy-induced apoptosis in an RB- and schedule-dependent manner. See: E. A. Sausville, Combining cytotoxics and 17-allylamino, 17-demethoxygeldanamycin: sequence and tumor biology matters, Clin. Cancer Res., 7: 2155-2158, 2001." Clin Cancer Res **7**(8): 2228-2236.
Nathan, D. F. and S. Lindquist (1995). "Mutational analysis of Hsp90 function: interactions with a steroid receptor and a protein kinase." Mol Cell Biol **15**(7): 3917-3925.
Nathan, D. F., M. H. Vos, et al. (1997). "In vivo functions of the Saccharomyces cerevisiae Hsp90 chaperone." Proc Natl Acad Sci U S A **94**(24): 12949-12956.
Nathan, D. F., M. H. Vos, et al. (1999). "Identification of SSF1, CNS1, and HCH1 as multicopy suppressors of a Saccharomyces cerevisiae Hsp90 loss-of-function mutation." Proc Natl Acad Sci U S A **96**(4): 1409-1414.
Obermann, W. M., H. Sondermann, et al. (1998). "In vivo function of Hsp90 is dependent on ATP binding and ATP hydrolysis." J Cell Biol **143**(4): 901-910.
Pace, C. N., B. A. Shirley, et al. (1996). "Forces contributing to the conformational stability of proteins." FASEB J **10**(1): 75-83.
Panaretou, B., C. Prodromou, et al. (1998). "ATP binding and hydrolysis are essential to the function of the Hsp90 molecular chaperone in vivo." EMBO J **17**(16): 4829-4836.
Panaretou, B., G. Siligardi, et al. (2002). "Activation of the ATPase activity of hsp90 by the stress-regulated cochaperone aha1." Mol Cell **10**(6): 1307-1318.

Pearl, L. H. and C. Prodromou (2000). "Structure and in vivo function of Hsp90." Curr Opin Struct Biol **10**(1): 46-51.
Pearl, L. H. and C. Prodromou (2006). "Structure and mechanism of the Hsp90 molecular chaperone machinery." Annu Rev Biochem **75**: 271-294.
Perutz, M. F. (1999). "Glutamine repeats and neurodegenerative diseases." Brain Res Bull **50**(5-6): 467.
Picard, D. (2002). "Heat-shock protein 90, a chaperone for folding and regulation." Cell Mol Life Sci **59**(10): 1640-1648.
Pimienta, G., K. M. Herbert, et al. (2011). "A compound that inhibits the HOP-Hsp90 complex formation and has unique killing effects in breast cancer cell lines." Mol Pharm **8**(6): 2252-2261.
Piper, P. W., S. H. Millson, et al. (2003). "Sensitivity to Hsp90-targeting drugs can arise with mutation to the Hsp90 chaperone, cochaperones and plasma membrane ATP binding cassette transporters of yeast." Eur J Biochem **270**(23): 4689-4695.
Pratt, W. B. and D. O. Toft (1997). "Steroid receptor interactions with heat shock protein and immunophilin chaperones." Endocr Rev **18**(3): 306-360.
Pratt, W. B. and D. O. Toft (2003). "Regulation of signaling protein function and trafficking by the hsp90/hsp70-based chaperone machinery." Exp Biol Med (Maywood) **228**(2): 111-133.
Prodromou, C., B. Panaretou, et al. (2000). "The ATPase cycle of Hsp90 drives a molecular 'clamp' via transient dimerization of the N-terminal domains." EMBO J **19**(16): 4383-4392.
Prodromou, C., S. M. Roe, et al. (1997). "Identification and structural characterization of the ATP/ADP-binding site in the Hsp90 molecular chaperone." Cell **90**(1): 65-75.
Ran, F., M. Bali, et al. (2008). "Hsp90/Hsp70 chaperone machine regulation of the Saccharomyces MAL-activator as determined in vivo using noninducible and constitutive mutant alleles." Genetics **179**(1): 331-343.
Retzlaff, M., F. Hagn, et al. (2010). "Asymmetric activation of the hsp90 dimer by its cochaperone aha1." Mol Cell **37**(3): 344-354.
Retzlaff, M., M. Stahl, et al. (2009). "Hsp90 is regulated by a switch point in the C-terminal domain." EMBO Rep **10**(10): 1147-1153.
Richter, K., J. Reinstein, et al. (2002). "N-terminal residues regulate the catalytic efficiency of the Hsp90 ATPase cycle." J Biol Chem **277**(47): 44905-44910.
Richter, K., J. Reinstein, et al. (2007). "A Grp on the Hsp90 mechanism." Mol Cell **28**(2): 177-179.
Roe, S. M., M. M. Ali, et al. (2004). "The Mechanism of Hsp90 regulation by the protein kinase-specific cochaperone p50(cdc37)." Cell **116**(1): 87-98.
Roe, S. M., C. Prodromou, et al. (1999). "Structural basis for inhibition of the Hsp90 molecular chaperone by the antitumor antibiotics radicicol and geldanamycin." J Med Chem **42**(2): 260-266.
Ronnen, E. A., G. V. Kondagunta, et al. (2006). "A phase II trial of 17-(Allylamino)-17-demethoxygeldanamycin in patients with papillary and clear cell renal cell carcinoma." Invest New Drugs **24**(6): 543-546.
Samuels, C. E., P. G. Robinson, et al. (1975). "Decreased inhibition of platelet aggregation by PGE1 in children with cystic fibrosis and their parents." Prostaglandins **10**(4): 617-621.

Scheibel, T., H. I. Siegmund, et al. (1999). "The charged region of Hsp90 modulates the function of the N-terminal domain." Proc Natl Acad Sci U S A **96**(4): 1297-1302.
Scheufler, C., A. Brinker, et al. (2000). "Structure of TPR domain-peptide complexes: critical elements in the assembly of the Hsp70-Hsp90 multichaperone machine." Cell **101**(2): 199-210.
Schirmer, E. C., J. R. Glover, et al. (1996). "HSP100/Clp proteins: a common mechanism explains diverse functions." Trends Biochem Sci **21**(8): 289-296.
Schmid, A. B., S. Lagleder, et al. (2012). "The architecture of functional modules in the Hsp90 co-chaperone Sti1/Hop." EMBO J **31**(6): 1506-1517.
Schulte, T. W., S. Akinaga, et al. (1998). "Antibiotic radicicol binds to the N-terminal domain of Hsp90 and shares important biologic activities with geldanamycin." Cell Stress Chaperones **3**(2): 100-108.
Sharma, S. V., T. Agatsuma, et al. (1998). "Targeting of the protein chaperone, HSP90, by the transformation suppressing agent, radicicol." Oncogene **16**(20): 2639-2645.
Shiotsu, Y., L. M. Neckers, et al. (2000). "Novel oxime derivatives of radicicol induce erythroid differentiation associated with preferential G(1) phase accumulation against chronic myelogenous leukemia cells through destabilization of Bcr-Abl with Hsp90 complex." Blood **96**(6): 2284-2291.
Soga, S., T. Kozawa, et al. (1998). "Radicicol leads to selective depletion of Raf kinase and disrupts K-Ras-activated aberrant signaling pathway." J Biol Chem **273**(2): 822-828.
Solit, D. B., I. Osman, et al. (2008). "Phase II trial of 17-allylamino-17-demethoxygeldanamycin in patients with metastatic melanoma." Clin Cancer Res **14**(24): 8302-8307.
Song, H. Y., J. D. Dunbar, et al. (1995). "Identification of a protein with homology to hsp90 that binds the type 1 tumor necrosis factor receptor." J Biol Chem **270**(8): 3574-3581.
Soti, C., A. Vermes, et al. (2003). "Comparative analysis of the ATP-binding sites of Hsp90 by nucleotide affinity cleavage: a distinct nucleotide specificity of the C-terminal ATP-binding site." Eur J Biochem **270**(11): 2421-2428.
Sreedhar, A. S., E. Kalmar, et al. (2004). "Hsp90 isoforms: functions, expression and clinical importance." FEBS Lett **562**(1-3): 11-15.
Stebbins, C. E., A. A. Russo, et al. (1997). "Crystal structure of an Hsp90-geldanamycin complex: targeting of a protein chaperone by an antitumor agent." Cell **89**(2): 239-250.
Stuhmer, T., A. Zollinger, et al. (2008). "Signalling profile and antitumour activity of the novel Hsp90 inhibitor NVP-AUY922 in multiple myeloma." Leukemia **22**(8): 1604-1612.
Supko, J. G., R. L. Hickman, et al. (1995). "Preclinical pharmacologic evaluation of geldanamycin as an antitumor agent." Cancer Chemother Pharmacol **36**(4): 305-315.
Tavaria, M., T. Gabriele, et al. (1996). "A hitchhiker's guide to the human Hsp70 family." Cell Stress Chaperones **1**(1): 23-28.
Taxis, C. and M. Knop (2006). "System of centromeric, episomal, and integrative vectors based on drug resistance markers for Saccharomyces cerevisiae." Biotechniques **40**(1): 73-78.

Terasawa, K., K. Yoshimatsu, et al. (2006). "Cdc37 interacts with the glycine-rich loop of Hsp90 client kinases." Mol Cell Biol **26**(9): 3378-3389.
Trepel, J., M. Mollapour, et al. (2010). "Targeting the dynamic HSP90 complex in cancer." Nat Rev Cancer **10**(8): 537-549.
Voellmy, R., A. Ahmed, et al. (1985). "Isolation and functional analysis of a human 70,000-dalton heat shock protein gene segment." Proc Natl Acad Sci U S A **82**(15): 4949-4953.
Wayne, N. and D. N. Bolon (2010). "Charge-rich regions modulate the anti-aggregation activity of Hsp90." J Mol Biol **401**(5): 931-939.
Weikl, T., P. Muschler, et al. (2000). "C-terminal regions of Hsp90 are important for trapping the nucleotide during the ATPase cycle." J Mol Biol **303**(4): 583-592.
Whitesell, L. and S. L. Lindquist (2005). "HSP90 and the chaperoning of cancer." Nat Rev Cancer **5**(10): 761-772.
Whitesell, L., E. G. Mimnaugh, et al. (1994). "Inhibition of heat shock protein HSP90-pp60v-src heteroprotein complex formation by benzoquinone ansamycins: essential role for stress proteins in oncogenic transformation." Proc Natl Acad Sci U S A **91**(18): 8324-8328.
Xu, Y., M. A. Singer, et al. (1999). "Maturation of the tyrosine kinase c-src as a kinase and as a substrate depends on the molecular chaperone Hsp90." Proc Natl Acad Sci U S A **96**(1): 109-114.
Young, J. C., V. R. Agashe, et al. (2004). "Pathways of chaperone-mediated protein folding in the cytosol." Nat Rev Mol Cell Biol **5**(10): 781-791.
Young, J. C., W. M. Obermann, et al. (1998). "Specific binding of tetratricopeptide repeat proteins to the C-terminal 12-kDa domain of hsp90." J Biol Chem **273**(29): 18007-18010.